REMARKS

IN THE CLAIMS

Claim 53 has been amended for greater clarity and particularity, to replace "said promoter" with "said expression control sequence". Support for that amendment can be found in Claim 31, wherein the antecedent "expression control sequence" (not "said promoter") appears.

I. Preliminary Remarks

Applicants gratefully acknowledge the Examiner's withdrawal of the 35 USC §101 rejection of Claims 39-40.

II. 35 USC § 112, First Paragraph (Written Description)

Claims 31-35, 39-40 and 53-55 stand rejected under 35 USC 112, ¶1 "as failing to comply with the written description requirement. . . . This is a new matter rejection." [Office Action page 3; emphasis in original.] More specifically, the Examiner states that the specification does not specifically describe "a vector that is capable of transcribing an antisense oligonucleotide in a human cell with a resultant decrease in the MN expression in the cell or claimed therapeutic effect of treating a neoplastic disease." [Office Action, at page 4; emphasis in the original.] Applicants respectfully traverse, arguing that a specification is interpreted according to what one

of ordinary skill in the art would understand is supported both explicitly and implicitly, and that the claims may be amended accordingly without adding new matter [MPEP §2163.07(a)].

First 35 USC §112, ¶1 Rejection is Improper: Rejection of Inherent Subject Matter as New Matter

Applicants respectfully submit that the first 35 USC §112, ¶1 rejection of the Office Action is improper as a "new matter" rejection of subject matter that is not "new matter" but instead constitutes subject matter inherent in the Specification. Applicants respectfully point out that it is axiomatic that the clarification of inherent subject matter does not add new matter to an application. [See, for example, In re Smythe, 178 USPQ 279 (CCPA 1973).]

The instant 35 USC §112, ¶1 rejection is based on a finding of lack of literal support in the Specification for the specific combination of elements individually described in the Specification:

> The specification, as originally filed, discloses antisense oligodeoxynucleotides that are complementary to MN mRNA sequence, wherein the antisense oligodeoxynucleotides are 19-mer or 29-mer (Example 10; Figure 3). It also discloses methods wherein naked antisense oligonucleotides (i.e., without being expressed by an expression vector) are added to the cell culture media. See Example 10. With regard to an expression vector, the specification discloses an antisense MN cDNA/promoter construct (page 65), wherein the

> full-length MN cDNA sequence comprising 1519 nucleotides in length is placed in an antisense orientation and the promoter is an MN promoter (page 40; Figure 15). Nevertheless, the specification is completely silent about a vector that is capable of transcribing an antisense oligonucleotide in a human cell with a resultant decrease in the MN expression in the cell or claimed therapeutic effect of treating a neoplastic disease.

[Office Action, bottom of page 3 to top of page 4; emphasis in the original.] Notably, the Examiner admits in the above passage that the Specification supports each of the individual limitations of Claim 31: 1) an MN antisense construct comprising a nucleic acid sequence from which an MN antisense nucleotide is transcribable; 2) wherein said nucleic acid sequence is operably linked to an expression control sequence in a vector; 3) wherein said MN antisense construct shows antisense activity in an in vitro screening assay; and 4) examples of MN antisense nucleotides that are MN antisense oligonucleotides capable of inhibiting MN gene expression. As it would have occurred to one of ordinary skill in the art at the earliest priority date to substitute fragments of antisense DNA in the place of full-length antisense DNA (cDNA), particularly in view of described MN antisense oligonucleotides that inhibited cell growth when added to the cells, Applicants respectfully argue that the claims only make explicit what one of skill in the art would understand from the implicit teachings of the Specification. "To comply with the written description

requirement of 35 USC 112, para. 1, . . . each claim limitation must be expressly, implicitly, or inherently supported in the originally filed disclosure." [MPEP §2163.05.]

MPEP at §2163.07(a) makes clear that a specification is interpreted according to what one of ordinary skill in the art would understand is supported both explicitly and implicitly, and that the claims may be amended accordingly without adding new matter:

> By disclosing in a patent application a device that inherently performs a function, operates according to a theory or has an advantage, a patent application necessarily discloses that function, theory or advantage, even though it says nothing explicit concerning it. The application may later be amended to recite the function, theory or advantage without introducing the prohibited new matter. *In re Reynolds*. . . 170 USPQ 94 (CCPA 1971); *In re Smythe* . . . [cited supra] (CCPA 1973).

As the PTO Board of Patent Appeals and Interferences stated in Ex parte Soreson, 3 USPQ2d 1462 (Bd. Pat. App. & Interf. 1987) at page 1463:

> [W]e are mindful that appellant's specification need not describe the claimed invention in *ipsis verbis* to comply with the written description requirement. . . . The test is whether the originally filed specification disclosure *reasonably* conveys to a person having ordinary skill that applicant had possession of the subject matter later claimed. . . .

[Emphasis in original.]

There is a clear basis in the original Specification for the claimed subject matter at issue, MN antisense oligonucleotide vectors. General support for MN antisense constructs comprising a nucleic acid sequence derived from the MN promoter can be found in the instant specification at the least at page 65, line 19 to page 66, line 25, which describes an "antisense" MN cDNA/MN promoter construct used to transfect CGL3 cells, particularly at page 66, lines 1-9. Support for the MN antisense nucleotide being an oligonucleotide of between 19 to 29 nucleotides in length, can be found at page 93, line 26 to page 94, line 5, which states: "Particularly preferred are the 29-mer ODN1 and 19-mer ODN2 for which the sequences are provided infra. Those antisense ODNS are representative of the many antisense nucleic acid sequences that can function to inhibit MN gene expression."

Moreover, as taught in the Mercola reference cited in the Specification at page 92, line 22 [Mercola, D., "Antisense fos and fun RNA," pp. 83-114, Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS, (Wiley-Liss, Inc., New York, NY, USA; 1991); copy enclosed], the preparation and utility of constructs expressing antisense fragments derived from the 5' region of c-*fos* or c-*jun* was known at the priority date. In particular, Mercola teaches that

> The use of antisense *fos* and *jun* RNA has contributed to our understanding of cell-cycle regulation, differentiation, gene regulation, and in particular, transformation. **In all effective cases, expression of antisense *fos* RNA via stable transfection of plasmids has led to a reduction of steady-state c-*fos* transcript levels** implying a mechanism of action that involves the breakdown of RNA involved in RNA duplex formation. **A wide**

> **range of sequences all containing 5′ portions of the c-*fos* gene have been used as a source of antisense RNA production** or DNA antisense oligonucleotide synthesis. **Similarly the 5′ coding region of c-*jun* has been used for the preparation of plasmids designed to express antisense RNA.**

[Mercola, at page 107, top of right column; emphasis added.]

Applicants respectfully point out that a "specification is directed to those skilled in the art and need not teach or point out in detail that which is well-known in the art." [In re Myers, 161 USPQ 668, 671 USPQ 668, 671 (CCPA 1969); see also, G.E. Col. V. Brenner, 159 USPQ 335 (CAFC 1968).] As the Federal Circuit stated in Spectra-Physics, Inc. v. Coherent, Inc., 3 USPQ 2nd 1737, 1743 (Fed. Cir. 1987): "A patent need not teach, and preferably omits, what is well known in the art." [Emphasis added.]

The Third Circuit stated referring to two Court of Customs and Patent Appeals (CCPA; predecessor court to the Federal Circuit) opinions [In re Wiggins, 179 USPQ 421, 424-425 (CCPA 1973) and In re Bode, 193 USPQ 12 (CCPA 1977)]:

> It is axiomatic that no description, however detailed, is "complete" in a rigorous sense. Every description will rely to some extent on the reader's knowledge of the terms, concepts, and depictions it embodies. Thus, an understanding of any description will involve some measure of inference. . . . [S]kill in the art can be relied upon to supplement that which is disclosed as well as to interpret what is written.

Rengo Co. Ltd. V. Molins Mach. Co., 211 USPQ 303, 319 (3d Cir. 1980). Applicants respectfully submit that the claimed subject matter would have been considered conventional to one of ordinary skill in the art, as it merely combines individual elements described in the specification (i.e., MN antisense oligonucleotides and MN antisense vectors comprising MN promoters), and as antisense oligonucleotide vectors were known in the art at the time of the earliest priority date (that is, October 21, 1992).

Antisense Oligonucleotide Vectors Were Conventional in the Art

Applicants respectfully submit that, at the priority date of the instant application (October 21, 1992), it was known how to successfully prepare vectors that expressed antisense oligonucleotides derived from that DNA sequence to inhibit the growth of neoplastic cells, once a DNA sequence of a critical gene had been identified. For example, De Benedetti et al. [Mol. Cell. Biology, 11(11): 5435-5445 (1991); copy enclosed] described experiments in which "HeLa cells were transformed to express antisense RNA [20 nucleotides in length] against initiation factor eIF-4E mRNA from an inducible promoter. . . . Induction of antisense RNA production was lethal." [Abstract].

De Benedetti et al. 1991 further provided extensive teachings as to how to make and use such antisense oligonucleotide

vectors to inhibit HeLa cell growth [infra]. De Benedetti et al. in turn relied on the teachings of others regarding the induction of antisense nucleotide expression [e.g., Holt et al., Proc. Natl. Acad. Sci. USA, 83: 4794-4798 (1986); Khokha et al., Science, 243: 947-950 (1989); McGarry et al., Proc. Natl. Acad. Sci. USA, 83: 399-403 (1986); see De Benedetti et al. at page 5442, middle of right column.] At least four other investigators (described infra under the response to the 35 USC 112, ¶1 enablement rejection), either successfully used antisense oligonucleotide vectors to inhibit tumor cell growth, or described their potential therapeutic use. Based on the above results, one of ordinary skill in the art would have considered that the technology of the antisense oligonucleotide vectors as described by De Benedetti et al. in 1991 could be applied to the MN gene as well.

Additional Argument: SEQ ID NO: 7

The Examiner also argues at page 4 of the Office Action that

> the specification does not describe SEQ ID NO:7 as a potential antisense oligonucleotide that decreases MN expression as claimed in clam 55; rather, it describes SEQ ID NO:7 only as a reverse primer sequence that is used in a RACE system for full-length MN cDNA cloning experimentation (page 39).

However, based on the efficacy of antisense oligonucleotide sequences ODN1 and ODN2 (SEQ ID NOS: 3 and 4) [Specification at page 24, lines 12-17 (Figure 3 description)], one of ordinary skill in the art would expect that antisense oligonucleotides having similar length and full complementarity to MN mRNA, such as SEQ ID NO: 7, would work as well, particularly in view of the above teaching from the Specification that ODN1 and ODN2 [SEQ ID NOS: 3 and 4] were "representative of the many antisense nucleic acid sequences that can function to inhibit MN gene expression . . ." [Specification at page 94, lines 3-5], and that

> [p]referred antisense oligonucleotides according to this invention are gene-specific ODNs or oligonucleotides complementary to the 5' end of MN mRNA. . . . Ones of ordinary skill in the art could determine appropriate antisense nucleic acid sequences, preferably antisense oligonucleotides, from the nucleic acid sequences of Figures 1A-1B, 15 and 25a-b.

[Specification, at page 93, line 24 to page 94, line 8.]

Applicants respectfully conclude that no new matter was added by the claimed subject matter which renders what was implicit in the Specification explicit in the claims. For the reasons provided above, Applicants respectfully request that the Examiner reconsider and withdraw the 35 USC §112, ¶1 "new matter" rejection of the pending claims.

III. 35 USC § 112, First Paragraph (Enablement)

Claims 31-35, 39-40 and 53-55 stand rejected under 35 USC 112, first paragraph,

> as failing to comply with the enablement requirement . . . .
>
> . . . .
>
> . . . [I]t would have necessitated an undue experimentation to construct a vector that is capable of expressing a short (19-29 mer) antisense oligonucleotide in a human cell or to use such vector to treat a neoplastic disease in a human . . . .

[Office Action, pages 4-7.] Applicants respectfully traverse, submitting that the initial burden of proof to challenge a presumptively enabling disclosure is upon the Examiner, and the Examiner has provided no objective evidence as to why the instantly claimed methods would not work. In addition, Applicants respectfully point out that at the time of filing an application, an applicant need not have any examples proving a claimed utility, and that the instant application provides references and examples in the Specification that support the construction of a vector that is capable of expressing short (19-29 mer) antisense oligonucleotides in a human cell and inhibit cell growth, as supported by the Gruenert Declaration under 37 CFR 1.132 filed on January 4, 2007. Furthermore, conventional art at the time of the priority date taught that antisense oligonucleotides produced by

vectors could overcome difficulties in uptake by cells and were effective at reducing tumor cell growth.

In re Marzocchi

The Federal Circuit quoted from In re Marzocchi, 169 USPQ 367, 369 (CCPA 1971) in In re Brana, 34 USPQ2d 1437 at 1441 (Fed. Cir. 1995) as follows:

> [A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support.

[Emphasis in the original.]

MPEP § 2164.04 entitled "Burden on the Examiner Under the Enablement Requirement" directs that the initial burden of proof to challenge a presumptively enabling disclosure is upon the Examiner. The patent case law, as well as the MPEP, makes clear that in accordance with case law, statements in a patent specification relied upon for enabling support that correspond in scope to a claimed invention "*must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of" those statements. [In re Marzocchi, supra; italicized emphasis in the original;

underlined emphasis added.] Applicants respectfully submit that there is no reason to doubt the objective truth of statements relied upon for enabling support in the Specification for the claimed invention.

Applicants respectfully point out that at the time of filing an application, an applicant need not have any examples. An invention may be constructively reduced to practice by filing an application with no working examples at all or with paper examples. As the Federal Circuit has stated:

> The first paragraph of § 112 requires nothing more than *objective* enablement. *In re Marzocchi*, . . . , 169 USPQ 367, 369 (CCPA 1971). How such a teaching is set forth either by the use of illustrative examples or by broad terminology, is irrelevant.

[In re Vaeck, 20 USPQ2d 1438 at 1445 (Fed. Cir. 1991); emphasis added.]

Incorporated References and the Gruenert Declaration

At page 6, the Office Action contends that "none of the references incorporated in the specification teaches, let alone insinuates, the feasibility or conception of cloning short antisense oligonucleotides into a vector." However, as pointed out supra, the Mercola reference cited at page 92 of the instant specification teaches that "[a] wide range of sequences all containing 5' portions of the c-*fos* gene have been used as a

source of antisense RNA production or DNA antisense oligonucleotide synthesis. Similarly the 5' coding region of c-*jun* has been used for the preparation of plasmids designed to express antisense RNA." Therefore, one of skill in the art was taught that antisense fragments expressed by a vector could be used to inhibit neoplastic cell growth.

Further, the Office Action states at page 6 that "nowhere in the 8-page [Gruenert] declaration [under 37 CFR 1.132 filed on January 4, 2007] is there a statement acknowledging that a plasmid or vector can successfully express MN antisense oligonucleotides in cells and confer therapeutic effects." However, Applicants respectfully point out that the Gruenert Declaration specifically cites, as support for the therapeutic efficacy of MN antisense oligonucleotides, the transfection experiments using MN antisense constructs described in the instant Specification. By citing those experiments, Dr. Gruenert implied that the technology and biological effects of MN antisense constructs was applicable to MN antisense oligonucleotides.

Dr. Gruenert states at page 3, Section 3(a) of the Declaration:

> I declare that the in vitro results shown in the subject specification, for example at pages 65-67, reasonably predict in vivo therapeutic efficacy of MN antisense oligonucleotides for the following reasons. First, there is a strong association of MN gene expression with tumorigenesis. Second,

> transfection experiments with MN sense and antisense constructs, in non-tumorigenic and tumorigenic cell lines, respectively, show that MN sense constructs cause non-tumorigenic cells to exhibit a transformed phenotype, whereas the antisense constructs cause the tumorigenic cells to have a very much lowered proliferation rate and to form smaller colonies than controls. Third, prior studies show that the in vitro effects observed in studies of other, structurally similar oligonucleotides, correlate with in vivo therapeutic effects.

Applicants respectfully note that Dr. Gruenert alternatively cites evidence from experiments using MN antisense oligonucleotides and MN antisense constructs as support for the utility of MN antisense oligonucleotides, implying that there no reason to think that the biological effects of MN antisense constructs did not also apply to MN antisense oligonucleotides. Further, at page 7, Section 8, Dr. Gruenert states: "I further declare that the published literature taught routine methods for designing, making, delivering, and evaluating oligonucleotides using for successful in vivo use." [Emphasis added.] Included in that "published literature" were the experiments of, for example, De Benedetti et al. 1992 [infra], which taught that the delivery of antisense oligonucleotides using constructs inhibited the growth of HeLa tumor cells.

In contrast, the Examiner has provided no evidence as to why one of ordinary skill in the art (at the time of filing) would expect the claimed subject matter not to work, and has only

provided evidence [bottom of page 5, Office Action] of earlier experiments using exogenous antisense oligonucleotides that did work [Zamecnik and Stephenson (1978); Stephenson and Zamenick (1978)], and later experiments using antisense oligonucleotide vectors that did work [Noonberg et al., Nucleic Acids Research, 22: 2830-2836 (1994)], which evidence only tends to support the enablement of the claimed methods.

Conventional Art at the Earliest Priority Date

Applicants respectfully submit that the Office Action is incorrect in stating at page 5: "Since Zamecnik and Stephenson first published their reports on antisense oligonucleotides that inhibit the targeted RNA in 1978 . . . , the effective delivery methods which utilize an expression vector and a promoter for expressing and producing short antisense oligonucleotides in mammalian cells were neither routinely practiced in the art nor practically conceived of in the art until the 1994 publication by Noonberg et al. . . ." Applicants respectfully submit that such effective delivery methods which utilize an expression vector and promoter for expressing and producing short antisense oligonucleotides were practiced in the art, not to mention practically conceived of in the art, at the priority date of the instant application, as evidenced at the least in the De Benedetti et al. 1991 article cited supra.

As discussed in the Section above in the response to the first 35 USC 112, ¶1 rejection, De Benedetti et al. 1991 [copy enclosed] described experiments which provided extensive teachings as to how to make and use vectors that expressed antisense RNA oligonucleotides [20 nucleotides] to inhibit HeLa cell growth. Substantial experimental detail is provided therein:

> The vector to express AS RNA [antisense RNA] against eIF-4E mRNA was constructed from RDB-DRE . . . by insertion of a double-stranded form of the antisense oligonucleotide into the *Xba*I site of the polylinker. *E. coli* JM110 cells were transformed, and plasmids from ampicillin-resistant colonies were screened for the presence of a *Cla*I site, which is created by the insertion of the antisense oligonucleotide.

[De Benedetti et al. (1991) at page 5436, Materials and Methods, middle of left column.]

> The vector used to express AS RNA [antisense RNA] was derived from BK virus and pSV-2neo. . . . **A sequence complementary to 20 nt near the 5′ terminus of eIF-4E mRNA was placed under control of the inducible promoter**. . . . Cells transformed with this vector (hereafter referred to as AS cells) grew slowly. . . . Addition of the inducer TCDD to AS cells caused further slowing of the growth rate and then a decline in cell number after 2 days (Fig. 1A). . . .
>
> . . . **These results indicate that the phenotype of slow growth is due to the expression of eIF-4E antisense sequences** and not to the vector per se, G418 or TCDD.

[Id. at page 5437, Results, bottom of left column to top of right column; emphasis added.] Figure 1A [id. at page 5437, top right

column] depicts growth curves of the antisense-expressing AS cells with and without added inducer TCDD: addition of TCDD to AS cells caused a clear decline in cell number.

De Benedetti et al. (1991) concludes:

> Melton . . . used AS RNA in *Xenopus* oocytes to inhibit β-globin translation and found that sequences complementary to the 5′ noncoding region or the 5′ region including the translation initiation site were more inhibitory than those complementary to the 3′ coding or 3′ noncoding regions. Izant and Weintraub . . . observed maximal inhibition of thymidine kinase expression in LTK- cells when they used AS RNA directed against the 5′ untranslated portion of the mRNA. On the basis of these results, we constructed the vector to express RNA which was complementary to 20 nt at the 5′ terminus of eIF-4E mRNA.
>
> . . . [T]he expression of AS RNA against eIF-4E mRNA produced marked effects in our system. The rate of cell division was slowed or stopped, depending on the level of expression (Fig. 1). . . .
>
> Another important feature of the system described here is the ability to regulate the level of AS RNA expression.

[Id. at page 5442, passage bridging left and right columns.]

Additional prior art also teaches the feasibility and efficacy of vectors expressing antisense oligonucleotides. For example, Joshi et al. [J. Virology, 65(10): 5524-5530 (Oct. 1991); copy enclosed], described retroviral vectors engineered to express 5′ oligonucleotide sequences of HIV-1 RNA in the antisense orientation and used to transform the human CD4+ lymphocyte-

derived MT_4 cell line. Cells expressing antisense RNA oligonucleotides to the 5′ sequence of HIV-1 RNA were resistant to HIV-1 infection. "These results indicate that sense and antisense approaches can be used to interfere with HIV-1 multiplication." [Joshi et al. (1991), last line of abstract at page 5524.]

Busch et al., [Boll. Soc. Ital. Biol. Sper., 67(8): 739-750 (August 1991); abstract enclosed] teaches that "[t]he epitope region [of G1-P120 antigen] contains the sequence Gln-Ala-Ala-Ala-Gly-Ile-Asn-Trp which is unique to the human P120 molecule; this may be a site for drug attack either by analogs to the region or by novel constructs based on antisense oligonucleotides." [Abstract, emphasis added; copy enclosed.] Nadkarni et al. [Medical Hypotheses, 35: 307-310 (1991); copy enclosed] suggests treating CML (a myeloproliferative clonal disorder of myeloid stem cells) by an antisense oligonucleotide construct which is introduced into CFU-GM cells (granulocyte-monocyte committed stem cells), and which transcribes a 16-20 nucleotide antisense RNA message which binds to the abnormal fusion mRNA expressed in the disease [see Abstract and page 309, top of left column.] Also, Raschella et al. [Cancer Res., 52: 4221-4226 (August 1, 1992); copy enclosed] describes the successful use of antisense vectors expressing fragments of human c-*myb* cDNA [224-bp and 231-bp in length] which inhibited the proliferation of transformed neuroectodermal cell lines, providing

proof that fragments of antisense DNA could be effective, in addition to entire cDNA sequences, in antisense vectors.

Based on the above results, one of ordinary skill in the art would have considered that the technology of the antisense oligonucleotide vectors as described by De Benedetti et al. (1991) and others was conventional art that could be applied to the MN gene as well.

Additional Arguments

The Examiner argues at the bottom of page 6 that "the specification does not teach that the naked MN antisense oligonucleotide interacts with MN gene at the DNA level as claimed in claim 35, although it shows the MN antisense oligonucleotide blocks MN transcript at the mRNA level." To the contrary, Applicants respectfully point out that the Specification teaches: "Antisense nucleic acid sequences substantially complementary to mRNA transcribed from MN genes, as represented by the antisense oligodeoxynucletides (ODNs) of Example 10, infra, can be used to reduce or prevent expression of the MN gene." [Specification, at page 92, lines 4-8]. One of skill in the art would consider that one mechanism by which antisense oligonucleotides could prevent MN gene expression would be direct binding to complementary DNA within the gene. One of ordinary skill in the art would also consider that the hybridization of such MN antisense

oligonucleotides to MN sense DNA to be within the scope of the invention, in view of the Specification's teachings that

> DNA or RNA having equivalent codons is considered within the scope of the invention, . . . as well as those nucleic acid sequences that would hybridize to said exemplary sequences [SEQ. ID. NOS. 1, 5 and 23] under stringent conditions or that but for the degeneracy of the genetic code would hybridize to said cDNA nucleotide sequences under stringent hybridization conditions.

[Specification, at page 37, lines 2-11.]

For the reasons provided above, Applicants respectfully conclude that the instant specification combined with conventional art provided sufficient enablement for the claimed subject matter. In view of the above remarks, Applicants respectfully request that the Examiner reconsider and withdraw the instant 35 USC §112, ¶1 enablement rejection.

35 USC § 112, Second Paragraph

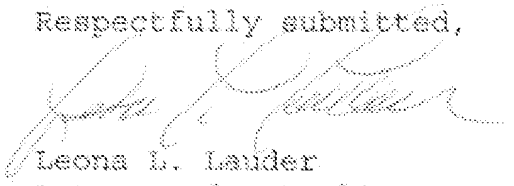
Claim 53 stands rejected under 35 USC § 112, second paragraph, as "[t]here is insufficient antecedent basis for this limitation ["said promoter"] in the claim." Applicants respectfully point out that Claim 53 has been amended to recite "said expression control sequence" instead, which phrase has antecedent basis in independent Claim 31 from which Claim 53 depends.

Applicants respectfully submit that Claim 53 now meets the 35 USC 112, second paragraph requirement for definiteness, and requests that the Examiner reconsider and withdraw the instant 35 USC 112, second paragraph rejection.

CONCLUSION

Applicants respectfully submit that Claims 31-35, 39-40 and 53-55 are in condition for allowance, and earnestly request that the claims be promptly allowed. If for any reason the Examiner feels that a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to telephone the undersigned Attorney for Applicants at (415) 981-2034.

Respectfully submitted,



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Dated: September 12, 2007

Antisense *fos* and *jun* RNA

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INTRODUCTION

The use of antisense *fos* RNA and, to a lesser extent, antisense *jun* RNA has contributed to our understanding of the roles of these gene products in cell cycle regulation, differentiation, transcriptional regulation, and, especially, transformation. In addition, recently the c-*fos* promoter itself has been found to be a target of negative regulation by the c-*fos* product and has been studied by use of antisense techniques; the topic is at present the subject of controversy. Preliminary studies of the differential roles of the various *fos* and *jun* family members and the use of *fos* ribozymes have been started. Progress in the application of antisense RNA and oligonucleotides to these topics and implications for diagnostic and therapeutic approaches are considered.

Here the convention of italicized three-letter acronyms for genes is followed; the genomic protein product is referred to by capitalized acronyms.

Fos and Jun have been reviewed recently (Vogt and Bos, 1989; Ovitt and Rüther, 1989; Morgan and Curran, 1989; Karin, 1990).

THE FOS AND JUN FAMILIES

There are five recognized cellular members of the Fos superfamily: c-*fos* (van Beveren et al., 1983), *fos*-B (Zerial et al., 1989), *fos*-D, Fos-related antigen 1 (*fra*-1) (Cohen and Curran, 1988), and Fos-related antigen II (*fra*-II) (Nishina et al., 1989). The corresponding gene products are variable in size, 275–367 amino acids, but exhibit overall homology and contain highly conserved regions with amino acid sequences indicative of particular structural motifs. The central ca. 85 amino acid portion contains a "leucine zipper" defined by a helix with a regular repeat of five leucine residues forming a hydrophobic surface which is known (Kouzarides and Ziff, 1988; Sellers and Struhl, 1989) to mediate interactions with a similar motif of Jun (Fig. 1). While Jun is capable of forming homodimers via interactions of this surface, none of the Fos family members forms homodimers but all form heterodimers with Jun (Chiu et al., 1988; Franza et al., 1988; Halazonetis et al., 1988; Kouzarides and Ziff, 1988; Nakabeppu et al., 1988; Rauscher et al., 1988; Sassone-Corsi et al., 1988; Zerial et al., 1989; Smeal et al., 1989). Thus the exact composition of the Fos–Jun complex, first purified from HeLa cells as activator protein-1 (AP-1)—a transactivation factor that mediates gene induction by phorbol acetate (TPA)—likely reflects the predominant Fos member(s) present in a particular cell type (Chiu et al., 1988; Rauscher et al., 1988; Karin, 1990). So far no gross differences in the DNA-binding properties of the various Fos–Jun complexes have been reported (Karin, 1990). Recognition of the general composition of AP-1 suggests that Fos or certain family members potentially mediate transactivation or repression of genes containing AP-1 or Fos–Jun complex binding sites such as α_1(III)collagen, collagenase, major histocompatibility complex (MHC) class I, or, for repression, heat shock protein 70.

Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS, pages 83–114

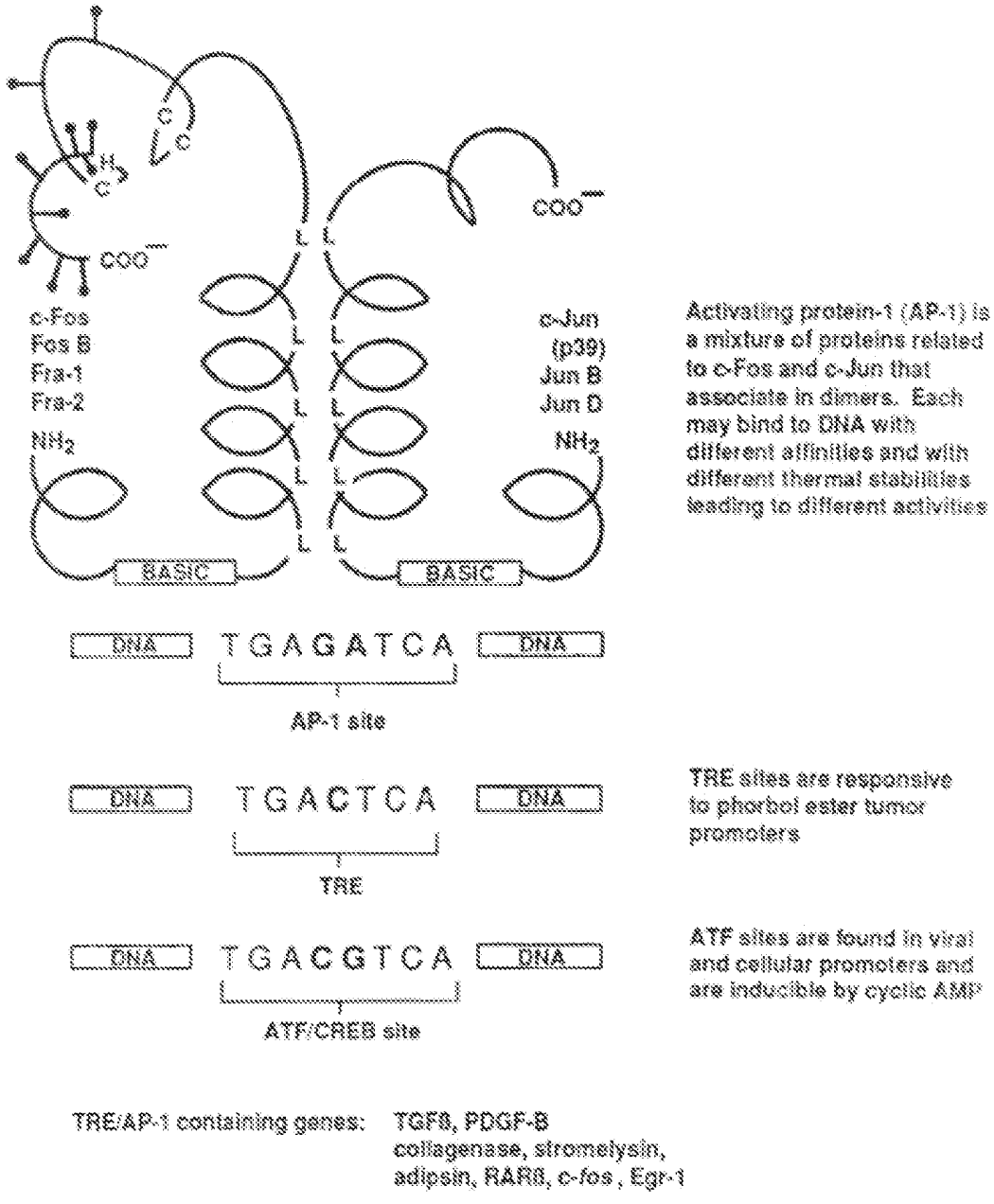

Fig. 1. Diagrammatic representation of structure of the Fos–Jun heterodimer and interaction with an "AP-1" site. The C-terminal portion of the Fos family proteins is rich in serine residues (—●) that undergo multiple posttranslational phosphorylations that may be important in regulation (Miller et al., 1984; Lee et al., 1988; Gaus et al., 1990) and has weak homology to a "zinc finger" (Mercola et al., 1986; Mölders et al., 1987). C, H, and L are single letter amino acid code showing the relative positions of the indicated amino acids.

On the N-terminal side of the leucine zipper is a second highly homologous region of ca. 45 amino acids containing numerous basic residues known to be important for DNA binding. The Fos family members have little or no DNA-binding affinity; however, the DNA-binding affinity of the Jun family members is greatly increased in the presence of any of the Fos family members (Chiu et al., 1988; Nakabeppu et al., 1988; Halazonetis et al., 1988; Zerial et al., 1989). Thus heterodimer formation is important for high affinity DNA binding consistent with the palindromic nucleotide consensus sequence (Fig. 1).

A major characteristic of the Fos family members is very rapid and transient increase in transcription in response to a large number of stimuli. Typically, peak transcript levels occur in 15–30 min and return to basal levels in 1–2 hr. The stimulatory effects can be understood in terms of the complex array of positive regulatory elements of the c-*fos* promoter (Fig. 3; for reviews see Ovitt and Rüther, 1989; Vogt and Bos, 1989). The rapid decline is due to at least three mechanisms: a short mRNA half-life of 15–30 min (Miller et al., 1984; Meijlink et al., 1985; Treisman, 1985; Rahmsdorf et al., 1987; Wilson and Treisman 1988; Bonnieu et al., 1988; Shyu et al., 1989), possible control at the level of transcription by a short-lived suppressor protein(s) (Miller et al., 1984; Greenberg et al., 1986; Lee et al., 1988), and control of the half-life of Fos itself possibly through phosphorylations of serine residues (Fig. 1) of the C terminus (Miller et al., 1984; Lee et al., 1988). Fos provides one example of a suppressor protein effecting negative autoregulation (Sassone-Corsi et al., 1988; Schönthal et al., 1989; see below).

Rapid, transient responses have been recognized in a large number of early growth response or primary response genes such Early Growth Response gene-1 (*Egr*-1) as discussed below (Figure 2).

There are at present three recognized cellular members of the Jun superfamily: c-*jun* (Bohmann et al., 1987; Angel et al., 1988), *jun*-B (Ryder et al., 1988), and *jun*-D (Ryder et al., 1989; Hirai et al., 1989). They contain broadly homologous regions necessary for dimer formation and DNA binding as described above. In addition the DNA-binding domain is homologous to the DNA-binding domain of the yeast transcription factor GCN4 and can functionally substitute for the domain of GCN4 in yeast (Struhl, 1988; Angel et al., 1989). Jun-B is a much weaker transactivator than c-Jun and indeed elevated concentrations of Jun-B antagonize activation by c-Jun. The effect is traceable to the N-terminal half of the molecule (Chiu et al., 1989). These and other observations (Schütte et al., 1989) strongly suggest that Jun-B is a

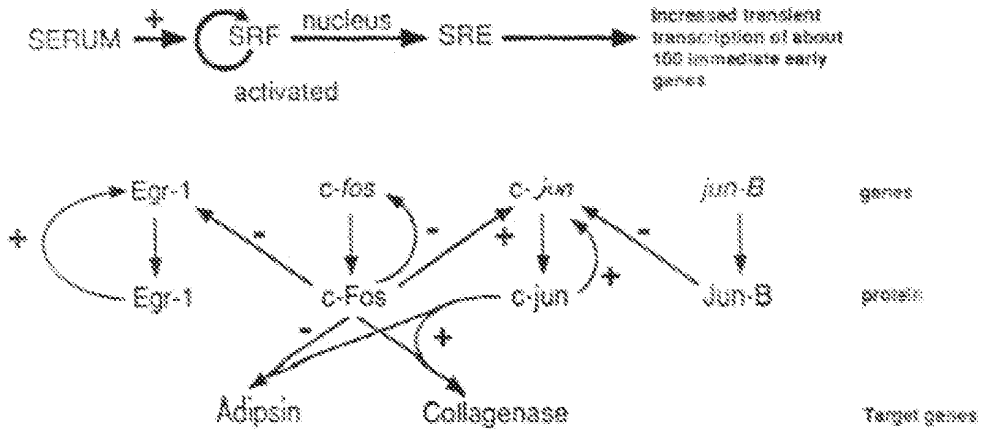

Fig. 2. Regulatory interrelations among the early growth response gene products Fos, Jun, and Egr-1.

negative regulator of c-Jun synthesis (Fig. 2) and may be responsible for the transient nature of the expression of c-*jun* (Karin, 1990). Thus the possibility exists of very different functions within the superfamilies (Fig. 2).

The antisense studies reviewed here have been carried out before and during the emergence of additional family members and therefore were not designed to discriminate among them. However, this is unlikely to be a complication. Successful application of the antisense method requires a high degree of complementarity both in extent of base pairing and in the length of contiguous pairs (Izant and Weintraub, 1985; Smith et al. 1986; Agris et al., 1986; McGarry and Lindquist, 1986; Chang et al. 1989). Cross-hybridization is unlikely with <70% identity. The sequences of the Fos and Jun family members show homology >70% only in the regions of putative DNA binding and leucine zipper formation. Virtually all sequences used for antisense studies considered here are derived from 5′ noncoding and first exon sequences that exhibit <45% homology. It is very unlikely that the antisense results involve significant cross-hybridization; however, this remains unproven.

c-*fos* IN CELL-CYCLE REGULATION

Serum Regulation of Fibroblasts

The potential transforming properties of protooncogenes including c-*fos* suggest roles in the regulation of proliferation (Bishop, 1985). This conclusion, together with the rapid response of c-*fos* to a variety of mitogenic agents such as platelet-derived growth factor (PDGF) (Greenberg and Ziff, 1984; Kruijer et al., 1984; Müller et al., 1984) led to the hypothesis that c-*fos* was important in the recruitment of quiescent fibroblasts into the cell cycle (Greenberg and Ziff, 1984; Kruijer et al., 1984; Müller et al., 1984). This view is supported by studies that show that the c-*fos* promoter (Fig. 3) contains several regions such as the serum response element (SRE) and *sis*-conditioned-medium element (SCM) that mediate serum and PDGF-like factor stimulation (Triesman, 1985; Hayes et al., 1987; Greenberg et al., 1987).

The hypothesis was studied in detail for NIH-3T3 cells using antisense techniques (Nishikura and Murray, 1987). Antisense c-*fos* (mouse) RNA was expressed via a plasmid containing nearly all of the first exon of c-*fos* together with 140 bp of 5′-noncoding DNA of the c-*fos* promoter region (Fig. 3). This sequence was placed in antisense orientation between two dexamethasone-inducible mouse mammary tumor virus long terminal repeats (MMTV LTRs). Multiple copies were introduced together with pSV2*neo*, as a source of neomycin drug resistance, by the calcium phosphate–DNA transfection procedure and multiple clones were selected by addition of the neomycin derivative G418. Resistant clones appeared with a low frequency of 10^{-6}—10-fold lower than for cells transfected with pSV2*neo* alone. A low frequency has been observed in other fibroblast-based systems (see "c-*fos* in transformation") and may be due to significant basal or "leaky" expression of antisense c-*fos* RNA, which interferes with an essential activity for growth. Thus the surviving clones may represent variants with lower basal expression of antisense RNA or possibly variants with alternate mechanisms of growth regulation.

Southern analysis of five clones revealed full-length insertion but with copy number varying from 1 to 40–50. One clone exhibited significant dexamethasone-dependent phenotypic changes and 6-fold decrease in steady-stage c-*fos* transcript levels. Consistent with this result, c-*fos* protein as judged by nuclear direct immunofluorescence studies was reduced in parallel with the decreased c-*fos* transcript levels. Antisense c-*fos* RNA production was confirmed. However, the levels were approximately 1% of sense levels and were not observed to be significantly increased by the addition of dexamethasone. Indeed for a clone with the single copy insertion and a clone with an aberrantly short antisense transcript, dexamethasone addition correlated with decreased steady-state antisense transcript levels. Thus there was poor correlation between

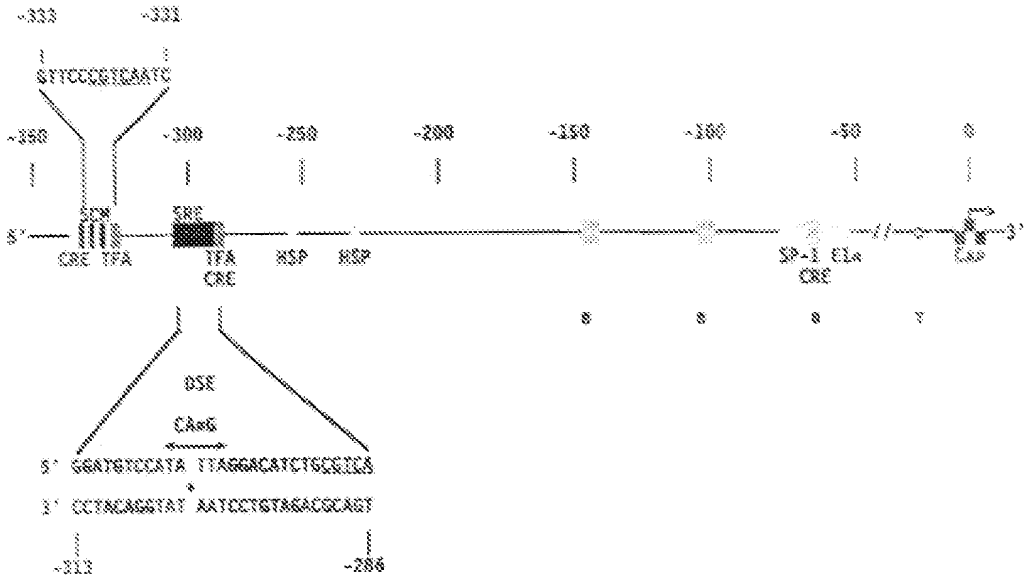

Fig. 3. Summary of the regulatory elements of the c-*fos* (mouse) promoter. TFA, AP-1-like binding site, underlined in expanded sequences; CRE, cyclic-like responsive element; SCM, *sis*-conditioned medium responsive element; SRE, serum response element or dyad symmetry element (DSE); E1a, adenovirus 2 E1a promoter homologous zone; HSP, heat-shock 70 promoter homology regions; SP-1, binding site for SP-1 transcription factor; B, sequences identified as important for regulation of basal expression; T, location of TATA box; ⊕, local 2-fold axis of symmetry relating palindromic sequences shown in bold.

copy number and dexamethasone stimulation. Antisense RNA even when polyadenylated has been observed to be unstable in mammalian cells (Izant and Weintraub, 1985). Recently it has been reported that RNA duplex degrading ("unwinding") activity is widespread in mammalian cells (Bass and Weintraub, 1988; Wagner and Nishikura, 1988; Wagner, 1989). It was concluded that the small amount of antisense RNA detected did not accurately reflect transcription (Nishikura and Murray, 1987). Duplex formation and fate were not described.

One clone of antisense regulated cells was used to examine the role of c-*fos* in the PDGF stimulated recruitment of quiescent cells into the cell cycle. DNA synthesis of serum-starved quiescent fibroblasts was monitored by autoradiography of [^{3}H]thymidine-labeled cells 24 hr after addition of purified PDGF in the presence or absence of dexamethasone. Antisense-regulated cells but not NIH-3T3 cells or control cells showed a dramatic decrease in the number of cells undergoing DNA replication when dexamethasone was present. The findings were supported by the results of a cell cycle distribution analysis. One additional clone with high copy number exhibited a substantial dexamethasone-dependent inhibition of PDGF stimulation. It was concluded that the large increase in c-*fos* that follows growth factor stimulation of quiescent cells is indeed a requirement for, not merely an accompaniment of, renewed growth.

For asynchronous populations of cells in the log phase of growth, induction of antisense c-*fos* expression was observed to have little effect, i.e., steady-state growth was normal under conditions in which enough antisense c-*fos* RNA is being expressed to inhibit the transition from quiescence to renewed growth. Therefore a distinction was made between the role of c-*fos* in the regulation of

growth renewal versus that for cycling cells. Indeed, only very low levels of c-*fos* transcripts are observed throughout the cell cycle for proliferating fibroblasts (Bravo et al., 1986).

Holt et al. (1986) examined the role of c-*fos* in cycling cells also using the MMTV LTR as a mechanism of inducible antisense RNA expression and noted a significant effect on cycling cells. Mouse c-*fos* DNA oriented in sense or antisense orientation was derived from a 301-bp fragment of mouse c-*fos* DNA that included 5′-noncoding sequences from just upstream of the cap site through most of the first exon (Fig. 4). Similar plasmids

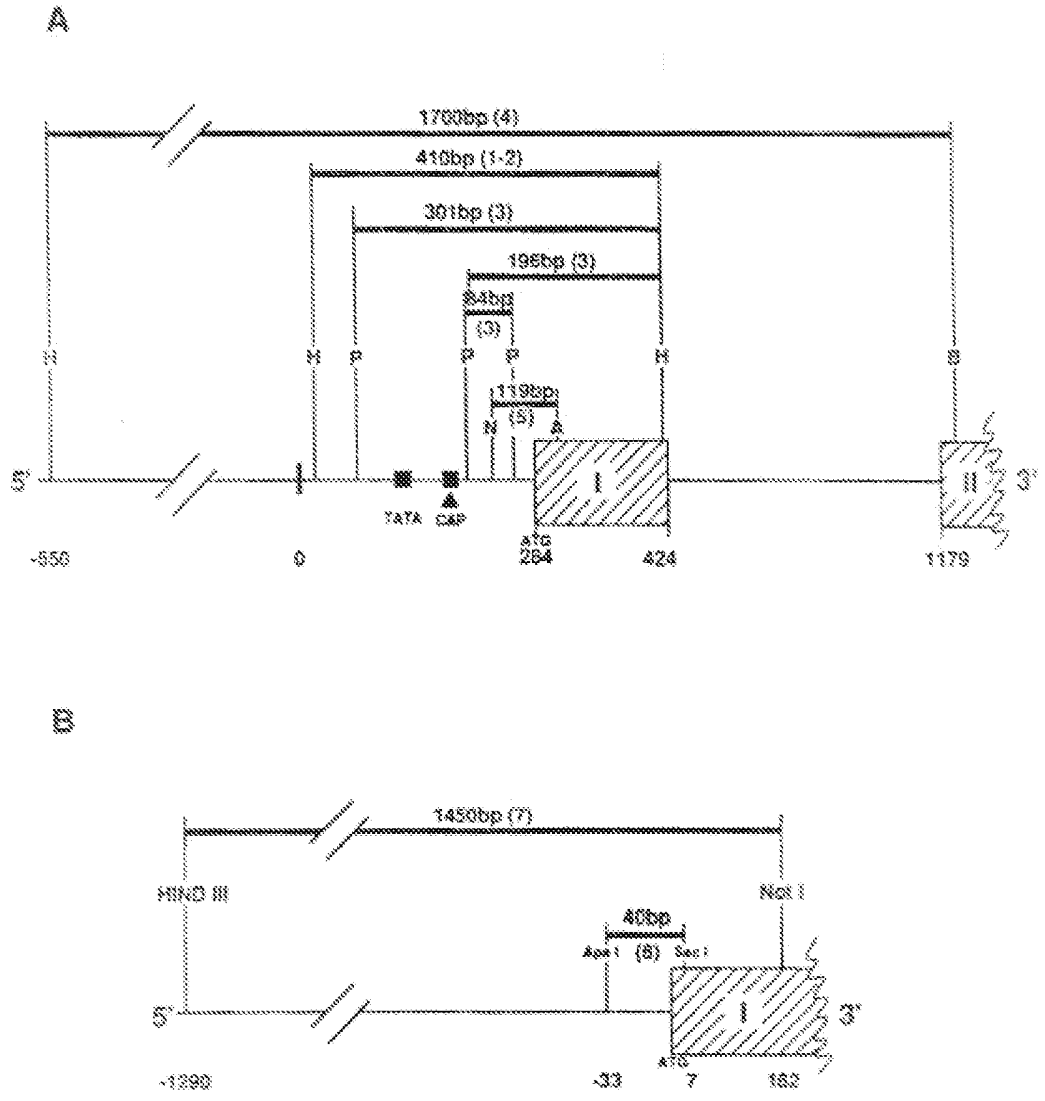

Fig. 4. Examples of sequences used for construction of plasmids expressing antisense *fos* (A) and *jun* (B) RNA. A, *Ava*I; B, *Bgl*II; H, *Hin*cII; N, *Nae*I; P, *Pst*I. References: 1. Nishikura and Murray (1987); 2. Levi and Ozato (1988); 3. Holt et al. (1986); 4. Mercola et al. (1988); 5. Schönthal et al. (1988a); 6. Schönthal et al. (1988b); 7. R. Chiu (personal communication; e.g., Kim et al., 1990). Not to scale.

based on a 196-bp fragment of the human c-*fos* sequence was also prepared. Finally a plasmid containing only an 84-bp 5′-noncoding fragment located between the cap site and the translation start site from mouse c-*fos* was also prepared. The *fos* sequences were inserted between the mouse mammary tumor virus (MMTV) promoter and a gene encoding β-globin such that detection of β-globin mRNA-containing transcript of the appropriate size for a fusion transcript was taken as an indicator of collinear antisense RNA production. Clones containing c-*fos* fragments in antisense orientation exhibited markedly decreased cloning efficiency. The effect was most marked for the construct with only noncoding c-*fos* DNA, 4% of a control nonfunctional plasmid. It was estimated that the surviving clones expressed up to 3000 copies of hybrid RNA per cell whereas the amount of c-*fos* target mRNA has been estimated as <5 copies per cell (Kruijer et al., 1984).

Proliferation studies of the resulting clones showed that the doubling times were reversibly increased 2- to 3-fold in the presence of dexamethasone. Again no correlation could be made between the amount of antisense c-*fos* RNA produced and the extent of growth inhibition—in fact maximum inhibition of growth was observed with clonal lines expressing 250–500 copies of antisense containing RNA per cell whereas a clone expressing 10-fold higher copy number exhibited an intermediate phenotype. No inhibition was observed for control clones expressing a similar copy number of sense-oriented transcripts. It was concluded that antisense *fos* RNA sequences inhibited clone formation and the growth of cycling cells in all clones that were isolated.

Several observations support a role for c-*fos* in cycling cells. Riabowal et al (1988) demonstrated that microinjection of *fos*-specific antibodies up to 6 to 8 hr after serum stimulation of quiescent rat embryo fibroblasts efficiently blocked DNA synthesis. Injection at later times had little effect, suggesting that c-*fos* function is required early in G_1. NIH-3T3 cells transformed either by v-*sis*, the homolog of c-*sis* that encodes the B-chain of PDGF, or transformed by activated EJ c-Ha-*ras* product exhibit rapid proliferation and loss of contact inhibition and are tumorigenic (Mercola et al., 1988; Ledwith et al., 1990). Constitutive expression of antisense c-*fos* RNA in v-*sis*-transformed NIH-3T3 cells leads to an 8-fold decrease in c-*fos* transcript levels compared to control cells and this effect correlates with a 2-fold decrease in log-phase growth rate (Mercola et al., 1987, 1988). As for PDGF, there is growing evidence that transformation by activated *ras* genes may work in part through activation of c-*fos*. Conditional expression of antisense c-*fos* RNA (using the antisense constructions of Holt et al., 1986) in EJ c-Ha-*ras*-transformed NIH-3T3 cells also led to a slower growth rate of exponentially growing cells (Ledwith et al., 1990). It may be therefore that in both these studies antisense c-*fos* RNA appears less efficient than antibody-mediated neutralization of c-*fos* protein where complete inhibition of growth was observed. The differences in efficiency may be less important in the regulation of steps requiring very low levels of Fos. However the transient accumulation of transcript levels required for the G_0 to G_1 transition is likely incomplete in antisense-regulated cells leading to decreased recruitment, a smaller growth fraction, and a decreased but finite growth rate.

Summary

Expression of antisense *fos* RNA in NIH-3T3 cells and derivatives leads to decreased steady-state c-*fos* transcript levels and prevents PDGF-stimulated accumulation of *fos* protein. Recruitment of quiescent cells into the cell cycle is restricted, strongly supporting an essential role of c-*fos* in the G_0 to G_1 transition. The doubling time of cycling populations is increased under conditions of antisense *fos* RNA expression and the combined evidence suggests that this is largely due to a role of c-*fos* at a second step in the cell cycle. These effects likely explain the markedly reduced cloning efficiency of cells bearing antisense *fos* RNA-expressing plasmids and conversely favor selection of clones

with alternate mechanisms of growth. There is poor correlation between the insertion copy number and the level of antisense RNA expression and between expression and phenotypic effect. Among the many factors involved may be suppressive effects at the site of insertion and idiosyncratic growth properties resulting from selection at reduced c-*fos* transcript levels, respectively. Thus there is considerable variation in the behavior of clones. A third potential role of c-*fos* on growth control is suggested by the possible inverse relation between c-*fos* transcript levels in transformed fibroblasts and sensitivity to density-dependent growth arrest discussed under "Transformation," below. Numerous mechanistic questions remain about the role of duplex formation in sequestering target RNA, promoting breakdown, inhibiting nuclear transport, or other steps necessary for interfering in mRNA function. The fate of target RNA, optimum concentration of antisense RNA, optimum site on target RNA, optimum size of antisense RNA, and related questions are incompletely understood.

F9 Embryonal Carcinoma (EC) Cells

EC cells are multipotent mouse cells derived from tumors of mouse embryonic tissue ectopically placed beneath the kidney capsule (for a review see Silver et al., 1983). Although termed "carcinoma," these cells can be stimulated to differentiate to a wide variety of ectodermal, endodermal, and mesenchymal tissues including aggregates of synchronously beating heart-like cells, skeletal muscle, and nerve-like derivatives. The best studied line, F9, is more restricted in its differentiation potential and is widely used as a model of differentiation.

The role of c-*fos* in growth regulation of F9 EC cells may be distinct from that of fibroblasts. Edwards et al. (1988) and Levi and Ozato (1988) introduced plasmids designed to continually express antisense c-*fos* RNA. In the construction of Levi and Ozato a 410-bp fragment of mouse c-*fos* containing the first exon and 5′ sequences similar to those used for the fibroblast studies (above) was inserted in antisense or sense orientation with respect to Rous sarcoma virus enhancer (RSV-LTR) (Fig. 4). Full-length plasmid insertion was confirmed by Southern analysis and full-length antisense RNA production was confirmed by Northern analysis. The efficacy of the constructions was tested by phorbol ester (TPA) and interferon challenges, which induce large and transient bursts of c-*fos* mRNA in F9 cells. C-*fos* expression was nearly eliminated in the antisense RNA expressing cells—the two examined clones exhibiting 35-fold and 20-fold lower levels of endogenous transcript than control cells. Thus, as with other studies (Nishikura and Murray, 1987; Mercola et al., 1987, 1988; Ledwith et al., 1990), antisense *fos* RNA expression is associated with decreased or abolished c-*fos* mRNA. However cloning efficiency of G418 resistant cells following transfection with these continuously expressing plasmids was not altered from that observed with sense control plasmids of $5–8 \times 10^{-5}$. Similarly, subsequent growth rates, although not quantitated, were described as comparable to that of untransfected cells.

Edwards et al. (1988) employed a similar fragment of c-*fos* mouse DNA as a source of antisense RNA in an SV40 early promoter-based vector. However, the vector was bifunctional, with the *fos* sequences inserted 3′ to the phosphotransferase gene of pNEO (Pharmacia). Following calcium phosphate-mediated transfection and selection (G418), full-length insertion was confirmed for several clones by Southern analysis and collinear transcription of both *neo* and antisense *fos* RNA was confirmed by sequential probing. C-*fos* transcript levels were shown to be substantially reduced—again illustrating that target RNA is largely destroyed in the presence of antisense c-*fos* RNA. Decreased Fos protein expression was also confirmed.

Cloning efficiency using the antisense *fos* expressing construction was high, 20×10^{-5}, although about 100-fold less than that for transfections using pSV2*neo* alone. Direct proliferation studies showed no differences in growth rate or final cell density for multiple clones of antisense expressing cells when

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These results contrast sharply with the very low cloning efficiencies of fibroblasts growing in the presence of antisense c-*fos* expressing constructions even when using the same promoter and antisense directing sequences in fibroblasts as used for the F9 cells (cf. Mercola et al., 1988 and Edwards et al., 1988). These results suggest that these cells utilize a growth mechanism that may be independent of c-*fos* or require comparatively less c-*fos* protein for cell cycle regulation. The conclusion is strongly supported by recent findings that, unlike most cell types, undifferentiated F9 stem cells do not contain Fos–Jun complex binding activity and basal c-*fos* expression is not detected (Angel et al., 1988; Chiu et al., 1988; Angel et al., 1989; Yang-Feng et al., 1990). Similarly c-*jun* and *jun*-B expression is very low or undetectable while that of *jun*-D is detectable but considerably lower than for differentiated cells (Yang-Feng et al., 1990). Thus the requirement for c-*fos* expression in growth may be cell type specific.

ANTISENSE *fos* AND *jun* RNA IN THE ANALYSIS OF THE AUTOREGULATION OF c-*fos*

Evidence for Autoregulation by Direct Binding by a Fos/Jun Complex

Little is known of the targets of *fos* protein action involved in cell cycle regulation; however, recent studies have revealed that one relevant target of c-*fos* transcription is its own promoter and that the interaction causes transcriptional repression (Sassone-Corsi and Verma, 1987). Subsequent studies, some based on antisense techniques, have led to contrasting results and the development of opposing mechanisms. At issue are whether additional factor(s) such as Jun cooperate with Fos, the identity of the DNA target sites, and which if any of the known interaction sites of the Fos protein are involved.

The autoregulatory mechanism was suggested by in vivo competition experiments that demonstrated that when DNA fragments of the c-*fos* promoter were cotransfected with a c-*fos* promoter–reporter chimeric gene based on the bacterial chloramphenicol acetyltransferase (CAT) gene as the reporter, a preexisting repression of promoter activity was effectively relieved (Sassone-Corsi and Verma, 1987). Additional cotransfection studies demonstrated that Fos expression vectors could suppress Fos–CAT synthesis (Sassone-Corsi et al., 1988). Direct measurement of endogenous c-*fos* transcript levels in HeLa cells showed that expression of mouse or human Fos protein suppressed the endogenous c-*fos* gene. However, repression by the v-*fos* product, which has a 3′ deletion leading to 49 C-terminal amino acids completely unrelated to those of Fos, was of low efficiency and a mutant of c-*fos* with a frame-shift 17 codons upstream of the viral deletion site did not repress the c-*fos* promoter at all. Promoter deletion experiments suggested that the target of repression resided in a region containing the 14-bp serum response element (SRE; Fig. 3). The SRE binds two proteins, the serum response factor (SRF) and p62, and possibly a third protein (Ryan et al., 1989). It was suggested that a complex of these proteins interacts with a Fos–Jun complex bound at the adjacent AP-1 site (Fig. 3). Thus a model of direct interaction of Fos with its own promoter was suggested.

Other studies using a combination of antisense techniques and transcription assays have confirmed and extended these observations (Büscher et al., 1988; Schönthal et al., 1988a, 1989). The basic paradigm for these experiments was to cotransfect NIH-3T3 cells with a Fos–CAT reporter gene and expression vectors for Fos or c-*fos* antisense RNA. In most studies CAT assays are carried out 10–24 hr after serum stimulation. Therefore in the simplest case the results represent the net effect of induction of Fos–CAT and repression by coinduced endogenous Fos. For example when quiescent NIH-3T3 cells were cotransfected with the Fos–CAT gene and a plasmid that expressed the human c-*fos* product, CAT synthesis was repressed to about one-third in comparison to cotransfection with a control plasmid (Schönthal et al., 1988a).

However cotransfection of the reporter gene with a plasmid bearing an antisense-oriented 119 bp fragment of mouse c-*fos* taken from the 5′-noncoding sequences and the first three codons of exon 1 under the control of an SV40 promoter (pSV*sof*), led to a 4-fold increase in CAT synthesis. These results suggest, first, that the *fos* promoter is specifically repressed in quiescent NIH-3T3 cells, presumably due in part to endogenous c-*fos* activity leading to the establishment of a low basal level of c-*fos* expression. Second, the *fos* promoter may be repressed further by exogenous Fos.

These results have been extended (Schönthal et al., 1989) to an analysis of the role of the Fos–Jun complex in growth factor signal transduction under conditions of mitogenesis. The results are based on an analysis of the consequences of serum stimulation of quiescent NIH-3T3 cells previously rendered quiescent, i.e., in "G_0" of the cell cycle, by maintenance of near-confluent monolayers in low (0.5–1.0%) serum for 1–2 days. Serum stimulation (addition to 20% fetal calf serum) of quiescent NIH-3T3 cells cotransfected with Fos–CAT and a control plasmid led to a 5-fold increase in CAT synthesis to saturating levels of the reporter system thus demonstrating the expected behavior of an intact c-*fos* promoter. However, when a continuously productive *fos* expression vector p8V*fos* was used in place of the control plasmid, basal CAT activity was reduced about 3-fold. Serum stimulation led to only about one-fifth the previous saturated rate—consistent with suppression of the reporter construct by the p8V*fos* product. When p8V*fos* was replaced by pSV*sof*, basal CAT activity rose over 10-fold. In this case serum stimulation again led to the saturated CAT synthesis rate. That the Fos–CAT results also reflect regulation of the endogenous c-*fos* promoter was confirmed by measuring c-*fos* transcript levels using an S1 nuclease protection analysis for cells stably transfected with MMTV-LTR-*fos* expression vector. Serum induction of c-*fos* transcription was markedly reduced in the presence of dexamethasone. Conversely, for cells stably transfected with an MMTV-LTR-*sof*, c-*fos* RNA expression is derepressed upon dexamethasone treatment. It was concluded that the low basal expression of c-*fos* and the rapid and effective turn-off of serum-induced c-*fos* transcription are due to autoregulation.

The participation of Jun was analyzed in analogous experiments (Schönthal et al., 1989; König et al., 1989). In this case cotransfection of quiescent NIH-3T3 cells with the Fos–CAT construct and a vector that continuously expressed Jun (pSV*jun*) again led to a 3-fold decrease in basal CAT synthesis. Serum stimulation led to increased CAT synthesis, which was, however, approximately 8-fold less than that seen with a control plasmid. Fos and Jun appeared to cooperatively repress the promoter since the use of both pSV*fos* and pSV*jun* in cotransfection studies led to over a 7-fold decrease in basal CAT synthesis (König et al., 1989).

In order to determine the effects of antisense *jun* RNA on this system a 40-bp synthetic oligonucleotide of the c-*jun* sequence starting 33 bp upstream from the translation start site was inserted in antisense orientation downstream of the SV40 promoter (Schönthal et al., 1988a). Cotransfection with this vector in place of pSV*jun* led to a 3-fold increase in basal transcription over the control value (Schönthal et al., 1989). As with antisense *fos* RNA expression, serum stimulation led to little or no additional effect, presumably owing to saturation of the reporter system. The results were interpreted to mean that in NIH-3T3 cells, the Fos product may exert its effect through formation of a Fos–Jun complex as has been shown previously for Fos-mediated transaction of AP-1 responsive genes (Chiu et al., 1988).

The above studies provide evidence that Fos and Jun function to repress the endogenous promoter following stimulation of mitogenesis. Deletion studies show that the major site of repression is the SRE (Lucibello et al., 1989; König et al., 1989; Shaw et al., 1989; Rivera et al., 1990; Guis et al., 1990) rather than the AP-1 sites (Fig. 3). Thus the SRE mediates both positive and negative effects. A number of examples of a single DNA regulatory site functioning in both positive

and negative regulation are known and their common features have been reviewed recently (Karin, 1990).

The AP-1 site adjacent to the SRE (Fig. 5) appears to mediate basal repression by the Fos–Jun complex. In order to demonstrate the physiological significance of basal regulation, again advantage was taken of p8V*sof* (Schönthal et al., 1988a). If the basal rate of transcription from the *fos* promoter was physiologically determined by the endogenous level of Fos and Jun proteins acting on or via the adjacent binding site, then antisense *fos* RNA should derepress the promoter. Cotransfection studies of p8V*sof* or a control plasmid together with a modified Fos–CAT with or without the AP-1 sequence were used to demonstrate derepression (König et al., 1989). The results were obtained under physiological conditions by carrying out nuclear run-on assays of NIH-3T3 cells that had been stably transfected with each Fos–CAT construct chimeric gene. Constructions with the DSE alone proved able to confer serum sensitivity. However, in the absence of the adjacent AP-1 site, the CAT transcription rate did not return completely to basal levels but retained 10–30% of the maximal rate (see also Shaw et al., 1989; Lucibello et al., 1989). Thus one determinant of the transient nature of the *c-fos* promoter activity was directly demonstrated. A model has been proposed by Shaw et al. (1989) suggesting that direct binding of the Fos–Jun complex at a site adjacent to the SRE can alter promoter activity by interacting with one or more of the bound proteins such as SRF and p62. Similar concepts have been proposed in several other promoters where DNA sequences immediately adjacent to regulatory elements mediate activating and suppressive effects such as for the interferon gene (Goodbourn and Maniatis, 1988) and the proliferin gene (Diamond et al., 1990).

In the above studies antisense expressing plasmids have been used to dissect a complex regulatory scheme. However, it should be noted that the mechanisms remain incompletely understood. Fos and Jun may affect repression by direct binding to the SRE–protein complex or CArg box (Fig. 3) (König et al., 1989; Lucibello et al., 1989; Guis et al., 1990); however, as yet complexes have not been detected (Rivera et al., 1990). An indirect mechanism is suggested by studies of the Egr-1 promoter, which contains multiple SRE-like sequences and CArG boxes. Repression by Fos appears independent of Jun since alteration of the amino acids essential for heterodimer formation have no effect. Alternatively, deletions in the C-terminal region led to complete loss of repression. The smallest deletion was the C-terminal 27 amino acids, which eliminated repression. These experiments have been confirmed and extended recently by a converse experiment in which an expression vector encoding a nuclear localization signal linked with the coding sequence of only the C-terminal 15 amino acids of Fos sequence was observed to confer repression (V. Sukhatme, personal communication). The sum of results favor an indirect mechanism possibly involving a Fos-induced repressor (Guis et al., 1990; V. Sukhatme, personal communication; cf. Rivera et al., 1990). It would be of interest to learn the effects of antisense *fos* and *jun* RNA expression in this system since, unlike the *c-fos* promoter, it would be expected that antisense *jun* RNA would have little effect while again antisense *fos* RNA would be expected to block repression.

Summary

Antisense *fos* and *jun* RNA has been employed as a complementary approach to the use of expression vectors in the analysis of gene regulation. In these studies antisense-induced loss of function was loss of a negative regulatory capacity and therefore led to a positive effect, namely increased promoter activity and gene expression. The observation of specific gene expression helps to allay worries that loss of function was simply the result of nonspecific inhibition of an essential cellular function by antisense RNA. However, the conclusion that an antisense RNA dependent mechanism accounts for the results rests with the biochemical consistency of the

results. Antisense RNA production was not confirmed, destruction or sequestration of target RNA or translation arrest of target product synthesis was not assessed, and controls utilizing sense-oriented DNA fragments or other specificity or toxicity checks were not described. All but one (Fig. 4) of the antisense *fos* RNA constructs described included the Kozak-like sequence, which for murine *c-fos* (5′-CGACCAUGA-3′) is similar to the consensus sequence [5′-CCAC-CAUG(G)-3′] associated with efficient translation of eukaryotic mRNA (Kozak, 1986) and so could potentially inhibit the synthesis of other proteins—perhaps repressors. It is of interest to note that a construct without complementary Kozak-like sequences exhibited prominent growth inhibitory properties (Holt et al., 1986; Ledwith et al., 1990).

The relative speed of transient transfection experiments has been made possible by combining a sensitive reporter gene downstream of sequences with positive or negative promoter regulatory properties, thus suggesting a method of some generality for analysis of gene regulation. A validation of the antisense mechanism occurring in transiently transfected cells will be necessary.

c-fos IN DEVELOPMENT

Several lines of evidence suggest that the Fos gene plays a role in differentiation and development. These include high expression in certain embryonic tissues (Adamson et al., 1983), the correlation of rapid transient expression in many cell types upon addition of agents that promote differentiation (Müller, 1983; Gonda and Metcalf, 1984; Greenberg et al., 1985; Curran and Morgan, 1985), and sustained expression during terminal differentiation of F9 EC cells (Edwards and Adamson, 1986). Consistent with these observations, transfection of F9 EC cells with Fos expression vectors leads to clonal lines that exhibit increased Fos protein levels and the appearance of Troma-1 and Troma-3—markers that accompany differentiation of F9 EC cells to parietal endoderm-like cells (Müller and Wagner, 1984; Rüther et al., 1985). However, there was poor correlation between the degree of expression of *fos* in a given clone and the expression of the markers. In addition, less than 10% of the cells of a given clonal line became differentiated by the criteria of Troma-1 and Troma-3 expression (Rüther et al., 1985). Further, the role of *c-fos* in differentiation is largely based on correlation data and has been challenged (Müller et al., 1985; Mitchell et al., 1986; Calabretta, 1987).

Two groups have employed antisense *fos* RNA techniques. Levi and Ozato (1988) and Edwards et al. (1988) examined the effects of the inducers retinoic acid (RA) and cyclic AMP (cAMP) on antisense-regulated F9 EC cells. When these cells are exposed to either RA or RA together with cAMP they are induced to differentiate over the following 4–8 days to cells with morphological and biochemical similarities to visceral and parietal endoderm, respectively. In both cases clonal populations of cells that constitutively express antisense *c-fos* RNA were examined and endogenous *c-fos* transcript levels where shown to be reduced or undetectable. Four days following stimulation of differentiation in either direction, Levi and Ozato (1988) observed morphological changes of antisense-regulated cells growing as attached cells in tissue culture. Further, the marker stage-specific embryonic antigen 1 decreased while the marker mouse histocompatibility complex (MHC) class I antigen increased, leading to the conclusion that, despite the profound inhibition of *c-fos* gene expression, clones expressing *c-fos* antisense RNA underwent differentiation after RA treatment. As a result of this observation, the expression of *c-fos* was examined in unregulated F9 EC cells and, similar to Mason et al. (1985) and Yang-Fen et al. (1990), no increase in *c-fos* transcript levels was observed to accompany differentiation during the first 2 days in contrast to previous results (Müller, 1983). However, timing and direction of differentiation may be important variables in understanding the role of *c-fos*.

Edwards et al. (1988) examined RA or RA-cAMP-induced differentiation using a variety

of markers specific for either parietal or visceral endoderm. In addition the F9 EC cells were grown as unattached and aggregated "embryoid bodies" that exhibit a striking morphological criterion in the form of a well-developed epithelial-like layer that forms in known stages over the course of 6 days. Endogenous *c-fos* expression in this system was in agreement with that of Lockett and Sleigh (1987), who showed that either RA alone or in combination with dibutyryl-cAMP induced high levels of *c-fos* mRNA levels in F9 EC cells that was maximal only after 6 days. Parallel experiments were carried out with six antisense *fos* RNA-regulated clonal lines bearing the bifunctional plasmid pSV*neosof* (cf. *c-fos* in "Cell-Cycle Regulation: F9 EC Cells," above). Two clones expressed reduced or absent levels of *c-fos* transcript and protein and were devoid of expression of laminin, type IV collagen, and proteoglycan-19 RNA transcripts after 4 days of induction with RA and dibutyryl-cAMP. Induction of Troma-1 and Troma-5 was also inhibited while control clones (pSV2*neo* only) behaved normally—in all suggesting inhibition of the formation of parietal endoderm. However, the antisense-regulated cells were not inhibited in the differentiation pathway to visceral endoderm since the α-fetoprotein gene was activated normally. The differential affect argues against an inhibition of parietal endoderm formation based on a nonspecific toxic effect associated with antisense *c-fos* RNA production or duplex formation. It was concluded that *c-fos* antisense expression effectively inhibits some aspects of differentiation in F9 cells and, moreover, the influence of *c-fos* on differentiation is specific to the parietal endoderm pathway.

The role of *c-fos* expression in the differentiation of rat pheochromocytoma (PC12) cells has also been examined (Kindy and Verma, 1988). Addition of nerve growth factor (NGF) to these cells in vitro leads to a large but transient increase in expression of *c-fos*, which is maximal in 30–60 min and returns to basal levels in about 2 hr. This is followed by the appearance of neurite-like outgrowths in several days. Addition of a synthetic 14-bp antisense DNA at 10μ*M* 4 hr prior to stimulation with inducer led to 80–100% inhibition of *c-fos* protein expression 50 min after addition of NGF. However, neuronal differentiation followed normally and it was concluded that NGF-stimulated *c-fos* expression was not necessary for NGF-stimulated differentiation of PC12 cells.

Summary

The combined results show that the use of antisense *fos* RNA has been effective in the analysis of differentiation. Many differentiation-inducing agents stimulate transient expression of *c-fos*; however, this appears to be unrelated to differentiation in many cases (e.g., Müller et al., 1985; Mitchell et al., 1986; Calabretta, 1987; Kindy and Verma, 1988). Elevated and continuous *c-fos* expression is required for the differentiation of F9 EC cells and this is specific to the parietal endoderm pathway.

THE FOS AND JUN FAMILIES IN TRANSFORMATION

Overexpression of *c-fos* causes transformation in susceptible cell types (Miller et al., 1984; Raymond et al., 1989) including primary fibroblast (Iba et al., 1988) and causes tumors in transgenic mice (Rüther et al., 1987, 1988, 1989). Antisense studies have implicated the continuous activation of *c-fos* in the mechanism of action of a wide variety of other protooncogenes including *c-sis*, c-Ha-*ras*, *c-mos*, and *v-src*. *C-fos* expression may be essential for the production of transformation-related products such as transin and stromelysin and *c-fos* amplification and increased expression have been associated with drug resistance of human colon carcinoma (Kashani-Sabet et al., 1990). *C-fos* is overexpressed in the majority of human osteosarcomas (Wu et al., 1990). Application of antisense has been useful in elucidating these relationships.

Sis

Overexpression of either *v-sis* (Favera et al., 1981; Devare et al., 1983; Huang et al.,

1984; Leal et al., 1985) or human c-*sis* (Clarke et al., 1984; Westin et al., 1984; Johnsson et al., 1985; Kelly et al., 1985; Stevens et al., 1988), which encode homologs of the B-chain of platelet-derived growth factor (PDGF), in established or primary fibroblast cell lines leads to complete transformation including tumorigenesis. Similar, albeit less dramatic results, hold for the A-chain of PDGF (Beckmann et al., 1988). Thus the observations of continuous or elevated expression of one or both of these products in a large number of human tumor types (Table 1) is provocative and suggests that one or more of the three possible isoforms of PDGF—consisting of AA, BB, or AB chain combinations—may play an important role in tumorigenesis (see Table 1 for references). Many of these tumor types have been shown to express one or both of the two cognate receptors of PDGF (Table 1). Thus autocrine stimulation of these receptors by PDGF molecules has been postulated as a mechanism of initiation and/or growth of these tumors (e.g., Garret et al., 1984; Graves et al. 1984; Betsholtz et al. 1984; Matsui et al. 1989). In view of the essential role of c-*fos* in PDGF-stimulated growth, it appears likely that c-*fos* activation may mediate *sis*-dependent transformation.

This hypothesis was tested by preparing stable clonal lines of v-*sis* transformed NIH-3T3 cells (SSV-3T3) bearing plasmids designed to continuously express antisense *fos* RNA (Mercola et al., 1987, 1988). The fragment consisted of a 1.7-kb portion of genomic mouse c-*fos* from the 5′-noncoding region and extending approximately half-way through the second exon, i.e., overlapping the antisense *fos* constructs described above. Control cells transfected with pSV2*neo* alone or with a plasmid containing a sense-oriented fragment continuously overexpressed c-*fos* by at least 8-fold—presumably due to continuous stimulation by the v-*sis* product. However, the antisense-regulated cells exhibited c-*fos* transcript and protein levels less than those of NIH-3T3 cells. The antisense-regulated cells exhibited several phenotypic criteria of reversal of transformation such as a flatter and larger cell morphology, decreased growth rate, and restoration of contact inhibition as judged by the reemergence of a saturation density with over 90% of the cells in G_0/G_1 of the cell cycle (Fig. 5). It was concluded that the overexpressed c-*fos* indeed functioned in the *sis*-dependent transformed phenotype to effect more rapid growth and loss of contact inhibition.

However, overexpression of c-*fos* does not appear to be sufficient for transformation. The antisense-regulated cells also exhibited anchorage-independent growth and tumorigenicity—albeit with a slow growth rate (Mercola et al., 1988). Thus either the SSV-3T3 parental cell line or transfected clonal lines had acquired additional transforming alterations, or there exist additional *sis*-dependent transforming pathways.

The confounding possibility of additional acquired genetic changes also arises when contemplating the significance of the *sis*-autocrine system apparent in the numerous and aneuploid human tumor lines (viz. Table 1). To distinguish among these possibilities we studied reversible transformation of NIH-3T3 cells bearing an inducible metallothionein–v-*sis* construct (Carpenter et al., 1989, 1991). These cells exhibited zinc-dependent and reversible transformation as judged by morphology, growth rate, loss of contact inhibition, and anchorage-independent growth (Fig. 6). Thus the *sis*-dependent pathway alone is sufficient for transformation in vitro. However, studies in vivo revealed two populations of tumor cells (Fig. 7). Cells subcultured from 10 of 18 tumors, all <0.1 g and ≤21 days in age, reverted to a normal phenotype by four criteria but could be induced to transform by the addition of zinc. Thus these tumor cells were also reversibly transformed, arguing that activation of the v-*sis* autocrine loop alone is sufficient for initiation of tumors. Cells from the remaining and larger, 0.5 ± 0.7 g, tumors did not revert by any criteria and therefore had become irreversibly transformed in vivo (Fig. 7). Southern analysis of tumor DNA suggested that there was no gross change in the pMT*sis* insert and Northern analysis of RNA from certain irreversibly transformed tumors demonstrated

TABLE 1. Expression of PDGF-Like Gene Products and Receptors in Selected Human Tumor Cell Lines and Tissues[a]

Cells and tissues		Receptor status	PDGF-like gene product
HT1080	Fibrosarcoma	ND	PDGF-A and -B (1–2)[b]
[illegible]	Glioma cell [illegible]	ND	PDGF-A only (3)
[illegible]	[illegible]	[illegible]	PDGF-B only (?)

TABLE 1. Expression of PDGF-Like Gene Products and Receptors in Selected Human Tumor Cell Lines and Tissues[a]

Cells and tissues		Receptor status	PDGF-like gene product
HTH080	Fibrosarcoma	ND	PDGF-A and -B (1–2)[b]
85-GT	Giant cell sarcoma	ND	PDGF-A only (4)
SKIMS	Leiomyosarcoma	α only (3)	PDGF-A only (4)
G402	Leiomyosarcoma	α only (3)	
RD	Rhabdomyosarcoma	ND	PDGF-A only (5)
A1186	Rhabdomyosarcoma	α > β (3)	
A204	Rhabdomyosarcoma	α only (3)	
A875	Melanoma	Not detected (3)	
WM266-4	Melanoma	ND	PDGF-A, short > long (6)
SW691	Melanoma	ND	PDGF-A only (7)
U2-OS	Osteosarcoma	PDGFR (8, 9)	PDGF-A > -B (4,8,10); PDGF-A, short ≥ long (6,11); PDGF-B > -A (9, see also 33)
Saos II	Osteosarcoma	ND	c-sis/PDGF-B only (4)
Saos	Osteosarcoma	ND	PDGF-B (5)
OS 1	Osteosarcoma	ND	PDGF-B not detected (5)
OS 2	Osteosarcoma	ND	PDGF-B not detected (5)
OS 3	Osteosarcoma	ND	PDGF-B (5)
TE	Osteosarcoma	ND	PDGF-B (5)
MG-63	Osteosarcoma	PDGFR present, not mitogenic (14)	Not detectable (15)
U1810	Osteosarcoma	ND	PDGF-A, short (16)
HOS	Osteosarcoma	ND	PDGF-A, short (16) PDGF-A, short only (11)
8842	Osteosarcoma	ND	PDGF-A not detected (16)
8SGT	Osteosarcoma	ND	PDGF-A, short > long (6)
T-2	Teratocarcinoma (clone 13)	ND	PDGF-B (17)
EJ	Bladder carcinoma	ND	c-sis/PDGF-B (18)
MCR-7	Breast carcinoma MDA-MB-231	ND	PDGF-A and -B (19)
T47D	Breast carcinoma	ND	PDGF-like factor (32)
	Breast carcinoma MDA-MB-157	ND	PDGF-like factor (32)
MCF-7	Breast carcinoma	Not detectable (21)	PDGF-A and -B (21)
BT-20	Breast carcinoma	Not detectable (21)	PDGF-A and -B (21)
ZR-75-1	Breast carcinoma	ND	PDGF-A and -B (21)
ABM	Ovarian carcinoma	Not detectable (21)	PDGF-A only (21)
OUN	Ovarian carcinoma	Not detectable (21)	PDGF-A and -B (21)
MAC	Ovarian carcinoma	ND	PDGF-A and -B (21)
SAM	Ovarian carcinoma	Not detectable (21)	PDGF-A and -B (21)

TABLE 1. *Continued*

Cells and tissues		Receptor status	PDGF-like gene product
A549	Lung carcinoma	Not detectable (21)	PDGF-A and -B (21)
CALU-1	Lung carcinoma	ND	PDGF-A and -B (21)
COLO201	Colon carcinoma	ND	PDGF-A and -B (21)
COLO205	Colon carcinoma	Not detectable (21)	PDGF-A and -B (21)
COLO357	Colon carcinoma	ND	PDGF-A>>-B (34)
WIDR	Colon carcinoma	ND	PDGF-B>>-A (34)
SW850	Pancreatic adenocarcinoma	ND	Not detected (34)
QGP1	Pancreatic adenocarcinoma	ND	PDGF-A>> -B (34)
Panc 89	Pancreatic adenocarcinoma	ND	PDGF-B>> -A (34)
Panc Tu1	Pancreatic adenocarcinoma	ND	PDGF-B>> -A (34)
Panc Tu2	Pancreatic adenocarcinoma	ND	PDGF-B>> -A (34)
ASPC 1	Pancreatic adenocarcinoma	ND	PDGF-A and -B (34)
BXPC 3	Pancreatic adenocarcinoma	ND	PDGF-A and -B (34)
HPAF	Pancreatic adenocarcinoma	ND	Not detected (34)
A590	Pancreatic adenocarcinoma	ND	PDGF-A>> -B (34)
PT 45P1	Pancreatic adenocarcinoma	ND	PDGF-A>> -B (34)
A-818-1	Pancreatic adenocarcinoma	ND	PDGF-A (34)
A-818-4	Pancreatic adenocarcinoma	ND	PDGF-A (34)
A-818-7	Pancreatic adenocarcinoma	ND	PDGF-A> -B (34)
KATO III	Gastric carcinoma	Not detectable (21)	PDGF-A and -B (21)
A431	Cervical squamous carcinoma	ND	PDGF-A, short > long (6) PDGF-B > -A (34)
HL-60	Myelocytic leukemia	ND	PDGF-A (18, cf. ref. 22)
U937	Promyelocytic leukemia	ND	PDGF-A (22)[c]
MOLT-4	T cell leukemia	ND	PDGF-A and -B (22)
K562	Erythroleukemia	ND	PDGF-A and -B (22)
A1690	Astrocytoma	β > α (28)	
A 172	Glioblastoma	β only (28)	c-sis/PDGF-B (11,12,18,23) PDGF-A, short only (11)
A2781	Glioblastoma	ND	c-sis/PDGF-B (18)
A1207	Glioblastoma	α only (28)	

	Relative mRNA[b]	
Receptor mRNA[a]	PDGF-A	PDGF-B

	Relative receptor mRNA[a]	Relative mRNA[a]	
		PDGF-A	PDGF-B
Malignant gliomas (3)			
U-87 MG	+	+ + +	+ +
U-105 MG	+ + +	+	–
U-118 MG	+ + +	+ +	(+)
U-138 MG	+ + + +	+	–
U-178 MG	+ + + + +	+ + + + +	+ +
U-251 MG(I)	–	(+)	+ + +
U-251 MGAg Cl. 1	–	+	+ +
U-251 MGsp	–	+	+ + +
U-343 MG	+(+)	+	–
U-343 MGa Cl. 2:6	–	+ + + +	+ + – +
U-343 MGa Cl. 2:6	ND	PDGF-A like (29)	
U-343 MGa Cls. 36L and 5H	ND	PDGF-A > -B (30)	
U-343 MGa Cl. 12:6 (D-1)	ND	PDGF-A >> -B (4)	
U-372 MG	+(+)	+ +	+ + + +
U-373 MG	ND	+	–
U-399 MG	+ +(+)	+ + +	+
U-410 MG	+ + + +	+	+
U-489 MG	+ + +	+ +	+
U-539 MG	+	ND	ND
U-563 MG	–	+	+
U-706 S	+	+	+ + +
U-1231 MG	+	+ + + +	+ + +
U-1240 MG	(+)	+ +	+ + + + +
U-1242 MG	+ + + +	+ + +	+
U-1706 MG	+(+)	+	+

	Receptor status	PDGF-like gene product
Primary cultures and biopsies		
Glioblastoma (n = 3)	β, 3 of 3 (28)	PDGF-A and -B, 3 of 3 (28)
Mesothelioma (n = 10)	ND	PDGF-A and -B (24)
Astrocytoma (n = 50)	PDGFR in 50% (31)	PDGF-A and -B in 86% (31)
Breast carcinoma (n = 4)	ND	PDGF-B in 3 of 4 (25)
Breast benign lesions (n = 2)	ND	PDGF-B by in situ hyb (25)
Fibrosarcoma (n = 4)	ND	PDGF-B in 3 of 4 (5)
Melanoma (n = 1)	ND	PDGF-B not detected (5)

TABLE 1. *Continued*

	Receptor status	PDGF-like gene product
Lymphoma, B cell ($n = 1$)	ND	PDGF-B (5)
WM9 Melanoma	ND	Undetectable (7)
WM115 Melanoma	ND	PDGF-A >> -B (7)
WM239A Melanoma	ND	PDGF-A >> -B (7)
WM266-4 Melanoma	ND	PDGF-A only (7)
SW691 Melanoma	ND	PDGF-A only (7)
Normal or immortalized cells		
Human diploid fibroblasts	α and β (3,26,27,35)	PDGF-A, cell-cycle dependent (14)
Murine diploid fibroblasts	α and β (3,26,27,35)	Undetectable (20)
Human umbilical vein endothelial cells		PDGF-A, short (16)

[a]Abbreviations used: PDGF-B = PDGF-B, transcript or protein product of *c-sis* gene; PDGF-A, transcript or protein product of the PDGF A-chain gene; PDGF-AB, heterodimer of PDGF A- and B-chains; PDGFR, PDGF receptor not specified as to subtype; α and β refer to PDGFR α and PDGFR β type receptor molecules; ND, not described. The PDGF A chain is specified as "long" or "short" depending on whether a 15 residue C-terminal fragment (GRPREGAAREARR) of exon 6 is known to be present or absent.

[b]References. 1. Eva et al. (1982); 2. Pantazis et al. (1985); 3. Matsui et al. (1989); 4. Bersbergen et al. (1986); 5. Fahrer et al. (1989); 6. Rorsman et al. (1988); 7. Westermark et al. (1986); 8. Betsholtz et al. (1984); 9. Nister et al. (1988a); 10. Heldin et al. (1986); 11. Yeng et al. (1987); 12. Harsh et al. (1989); 13. Paulsson et al. (1987); 14. Wenner et al. (1987); 15. Graves et al. (1986); 16. Collins et al. (1987); 17. Westra et al. (1989); 18. Igarashi et al. (1987); 19. Bronzert et al. (1987); 20. Bowen-Pope et al. (1984); 21. Sarthan et al. (1988); 22. Antalis et al. (1987); 23. Pantazis et al. (1985); 24. Versnel et al. (1990); 25. Ito et al. (1989); 26. Heldin et al. (1988); 27. Hart et al. (1988); 28. Hermansson et al. (1986); 29. Nister et al. (1988b); 30. Nister et al. (1987); 31. Maxwell et al. (1989); 32. Rosengart et al. (1985); 33. Graves et al. (1986); 34. Kahlhoff et al. (1991); 35. Bywater et al. (1988).

[c]PDGF-B expression is inducible by addition of the differentiation promoting phorbol esters to either HL-60 or U-937 cells (18,22).

[d]Arbitrary units of Nister et al. (1988a).

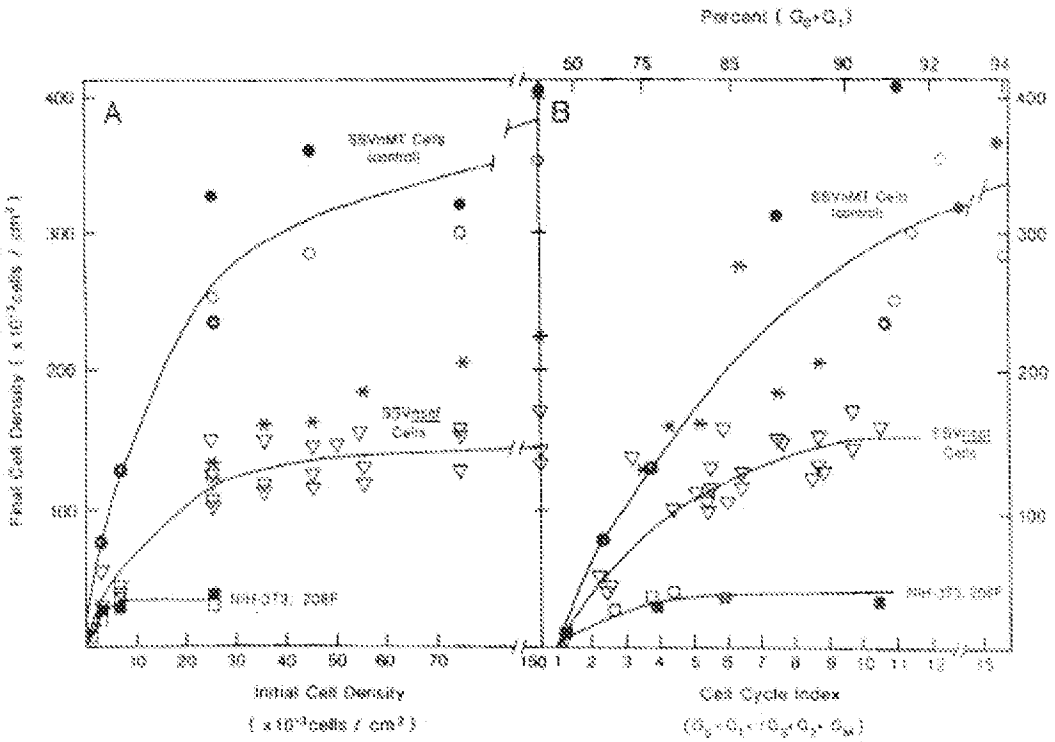


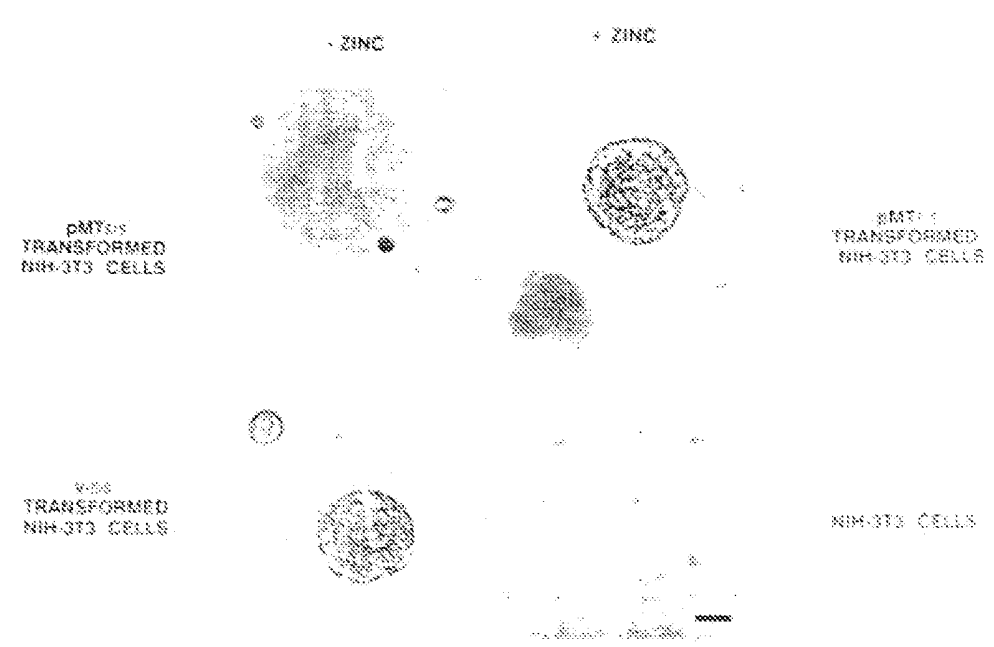
Fig. 5. Antisense *fos* regulated *v-sis*-transformed cells exhibit restoration of contact inhibition. (**A**) The *Y* axis gives the final saturation density obtained following plating at the density shown on the *X* axis and allowed to grow through log-phase growth, 4 days. NIH-3T3, parental cells; SSVnMT, control cells (*v-sis*-transformed NIH-3T3 cells transfected with p8V2*neo* and an irrelevant plasmid; solid curves are average of five clones) and SSV*nasf* antisense regulated cells (solid curves are average of six clones). Note that antisense-regulated cells do not grow above 150,000 cells/cm^2 even if seeded at that density 4 days prior to the measurement, unlike transformed control cells that achieve a postlog-phase density proportional to seeding. (**B**) The fraction of cells in G_1/G_0 of the cell cycle as a function of final saturation density. Note that for any density (*Y* axis) antisense-regulated cells exhibit a higher proportion of quiescent cells than the corresponding control cells. Reproduced from Mercola et al. (1988), with permission of the publisher.

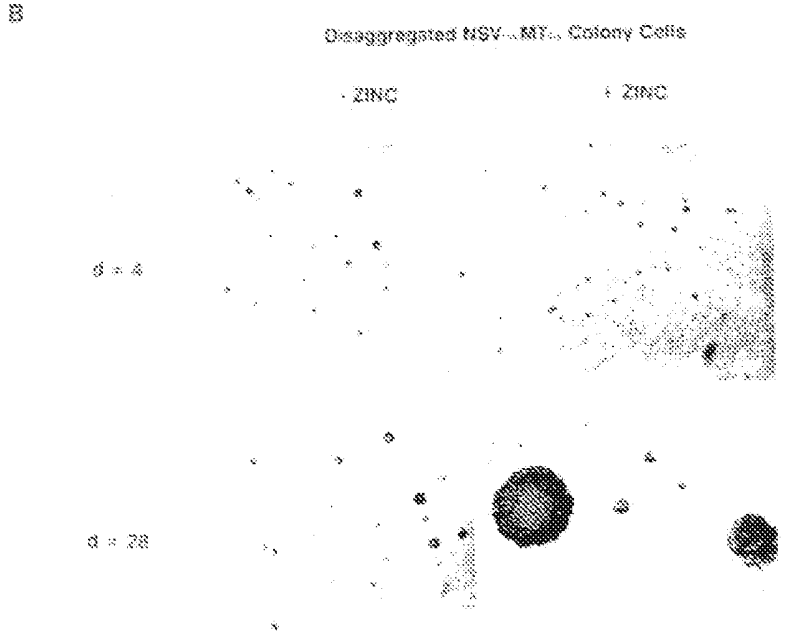
that zinc-dependent pMT*sis* expression remained intact. The combined observations suggest that extended tumor growth is accompanied by acquisition of other irreversible change(s) that occur at high frequency in vivo. Thus an activated *sis* gene may initiate a multistep process.

For the many human tumor types that continually express c-*sis* (i.e., Table 1), it may be possible, as our data suggest, that autocrine stimulation is related to initiation and may contribute to growth, but that additional genetic changes are likely to occur and contribute to the phenotype by the time these tumors are clinically evident. Thus a multistep (Fearon and Vogelstein, 1990) model best accounts for the sum of results.

c-Ha-*ras*, v-*mos*, and v-*src*

In studies carried out in parallel to the analysis of c-*fos* autoregulation, Schönthal et al. (1988a) observed that NIH-3T3 cells cotransfected with a reporter construct containing the AP-1 binding sequence of the human collagenase promoter together with prospective activators of c-*fos* such as MMTV-LTR-c-Ha-*ras*, -*mos*, or -*src* exhibited prominent and dexamethasone-dependent CAT


A
- ZINC
+ ZINC
pMT-v-src TRANSFORMED NIH-3T3 CELLS
pMT-c-src TRANSFORMED NIH-3T3 CELLS
v-src TRANSFORMED NIH-3T3 CELLS
NIH-3T3 CELLS


B
Disaggregated NSV-MT-1 Colony Cells
- ZINC
+ ZINC
d = 4
d = 28

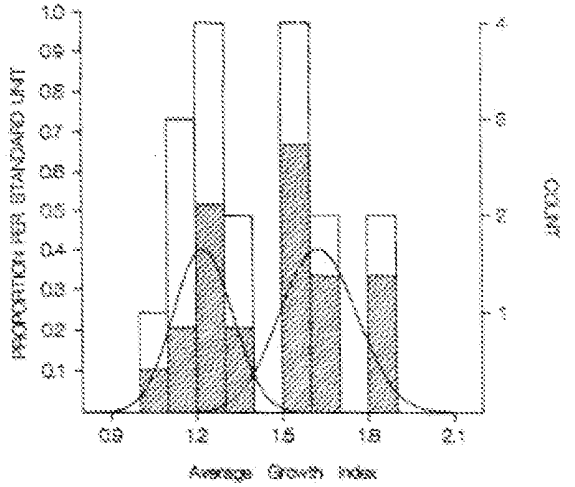


Fig. 7. The frequency distribution of the growth index of tumor cells is bimodal. Tumors of pMTs*is*-transformed cells were recovered from athymic mice and subcultured in the presence and absence of zinc. Reversibility of growth was determined by measurement of zinc induction of growth (growth index, GI)—defined as the total number of cells observed after log-phase growth (typically day 5 after seeding at 125,000 cells/cm^2) in the presence of zinc to that in the absence of zinc. The average GI of four independent clones of low passage pMTs*is*-transformed cells was 1.83 ± 0.2, whereas the average GI of control clones as well as the NIH-3T3 parental population was 1.0 ± 0.1. Approximately half of the tumor subcultures grew as fast as transformed cells in the absence of zinc, yielding an average GI of this of 1.22 ± 0.1, whereas the remaining tumor-derived cells reverted to a normal phenotype with normal morphology and growth but exhibit prominent zinc-dependent increase in growth with an average GI of 1.65 ± 0.13. The difference is highly significant, $P < 0.005$. Hatched bars, left ordinate; open bars, right ordinate.

synthesis. CAT synthesis increased up to a third that observed when Fos was overexpressed by pSV*fos* while nonproductive *fos* mutants and irrelevant plasmids had no effect. The three oncogene expression vectors separately activated a c-*fos* promoter in a dexamethasone-dependent manner up to 5-fold. To determine whether activation of the collagen promoter occurred via activation of the endogenous c-*fos* gene, stable cell lines containing each of the oncogene constructs were transfected with the collagen promoter–CAT construct with or without pSV*sof* and treated with dexamethasone. The antisense condition was associated with 1.7- to 3.2-fold less dexamethasone-stimulated activation. It was concluded that c-*fos* plays a key role in signal transduction by these agents. Further support derives from the observation that revertants of *fos*-transformed fibroblasts

◀Fig. 6. Anchorage-independent growth of conditional v-*sis* transformation is reversible. (A) NIH-3T3 cells stably transfected with pMTs*is* (NSVneoMTs*is* cells) exhibit zinc-dependent growth in methylcellulose, day = 12 after suspension. (B) Colonies recovered from methylcellulose and disaggregated by treatment with trypsin again exhibit zinc-dependent growth in soft agar illustrating that viable and mitotically active cells were recovered. However, no proliferation occurs after 4 weeks in the absence of zinc, arguing that no irreversible transforming events have occurred during *sis* expression and anchorage-independent growth, cf. **A**. Bar = 50 μm.

cannot be retransformed by the *fos*, *ras*, or *mos* oncogenes (Zarbl et al., 1987).

EJ c-Ha-*ras*-transformed NIH-3T3 cells have been studied in more detail (Ledwith et al., 1990). Using the constructs of Holt et al. (1986), stable transfected clones of the *ras* transformed cells were isolated and examined. As for SSV-3T3 cells (Mercola et al., 1988), antisense *fos* RNA expression led to a decrease in sense mRNA expression, morphological change, slower growth, and restoration of contact inhibition, and in addition exhibited inhibition of anchorage-independent growth (similar effects of antisense *fos* RNA in *ras*-transformed cells are quoted by Schönthal et al., 1988a). Although tumors of the antisense-regulated cells formed in athymic mice, in the presence of dexamethasone, a marked inhibition of tumor growth rate was observed. It was concluded that c-*fos* expression is required for *ras* transformation. The results are consistent with other studies implicating c-*fos* in the *ras* pathway (Stacey et al., 1987; Kerr et al., 1988).

Finally it is noted that, as for antisense *fos* RNA expression in *sis*-transformed cells (Mercola et al., 1988), decreased c-*fos* expression was associated not only with morphological and growth rate properties but also restored density dependence of growth arrest, suggesting the possibility that elevated c-*fos* levels antagonize the mechanism by which cell–cell contact inhibits cell division at characteristic cell densities.

Jun Family

Transactivation by Fos implies complex formation with a member of the Jun family since Fos alone has little of no DNA binding properties (Chiu et al., 1988; Halazonetis et al., 1988; Kousarides and Ziff, 1988; Nakabeppu et al., 1988; Rauscher et al., 1988; Sassone-Corsi et al., 1988; Zerial et al., 1989; Smeal et al., 1989). Thus it might be expected that certain of the Jun family members may also mediate signal transduction of the same oncogenic pathways. Antisense methods have been employed to explore the differential roles of the Jun family members in regulation of the interleukin-2 (IL-2) gene (M. Karin, personal communication). Activators of the IL-2 gene of EL4 cells—transformed murine T cells—strongly induce expression of *jun*-B but not c-*jun* or *jun*-D and strongly induce c-*fos* and *fos*-B but not *fra*-1. Addition of synthetic conventional DNA oligonucleotides complementary to 18 residues of the 5′-coding regions led to differential effects: antisense DNA complementary to *jun*-B or *fos*-B inhibited activation of the IL-2 promoter while antisense *jun*-D or c-*fos*, which strongly inhibited expression from a reporter construct containing three copies of a consensus AP-1 site, had no effect of expression of the IL-2 construct. The results suggest that Jun-B/Fos-B heterodimers and/or Jun-B homodimers are the preferred activators in this system. The results also suggest the possibility of differentiating the roles of the family members by use of antisense methods.

As noted, previous studies have suggested that elevated levels of Jun-B may antagonize activation by the c-*jun* product, possibly due in part repression of the c-*jun* enhancer (Chiu et al., 1989; M. Karin, personal communication). Similar results have been observed in 3T3 mouse fibroblasts, SK-Br-3 human mammary carcinoma cells, and PC12 cells (Brysch and Schlingensiepen, 1991; W. Brysch and K.-H. Schlingensiepen, personal communication). Growth studies were carried out for 5 days following addition to 2 μ*M* phosphorothioate DNA oligonucleotides compelementary to 14 nucleotides of the target sequence to freshly seeded (1 hr) cells. In all cases treatment with antisense to *jun*-B led to increased growth, which usually peaked in 1 to 2 days. Treatment with antisense to *jun*-D to the transformed cells led to a small increase in growth, which was maximal in 1 day while for 3T3 cells the stimulation was similar to that with seen with antisense to *jun*-B. Treatment with antisense to c-*jun* led to decreased growth in 3T3 and SK-Br-3 cells compared to cells treated with an oligomer with a randomized sequence. Further, addition of antisense DNA complementry to the p53 gene sequence to 3T3 or SK-Br-3 cells also led to increased growth maximal on days 1–2. The results were taken to suggest that

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Stromelysin/Transin

Transin is one of a family of metal-dependent proteases secreted by many transformed cells and may mediate metastasis via breakdown of collagen and extracellular matrix proteins (for a review see Matrisian and Hogan, 1990). Transin is induced by PDGF, epidermal growth factor (EGF), v-*fos*, H-*ras*, v-*src*, and others depending on cell type, suggesting a role for c-*fos*. McDonnell et al. (1990) examined stably transfected NIH-3T3 cells bearing the dexamethasone-inducible constructs of Holt et al. (1986). PDGF-induced transin transcript levels were nearly eliminated in the presence of dexamethasone, whereas in the absence of the steroid or with EGF, transin induction occurred normally, suggesting that both *fos*-dependent and *fos*-independent pathways for the two growth factors exist in NIH-3T3 cells. EGF-dependent induction of transin has been examined in rat-1 cells using synthetic antisense *fos* and *jun* DNA complementary to the first 18 coding nucleotides as well as the antisense RNA-expressing plasmids. EGF stimulation caused a transient increase in c-*fos* and c-*jun* transcript level, which was followed by the appearance of transin transcripts. However, when serum-deprived cells were first treated with antisense DNA (0.5 μM), immunoreactive Fos and Jun were eliminated and transin transcription was reduced by a third to a half. Sense control DNA had no effect. Similarly a 50% decrease in EGF-induced transin mRNA levels was observed for stably transfected cell lines bearing antisense *fos* RNA expressing plasmids. In support of these findings, Kerr et al. (1990) observed a novel binding site in the transin promoter capable of binding Fos induced in vivo by EGF.

Transforming Growth Factor-β1 (TGF-β1)

TGF-β1 has numerous effects, including growth-retarding effects in many cells (for a review see Moses et al., 1990). c-*fos*-dependent inhibition of EGF induction of transin (Kerr et al., 1990), and positive induction of its own promoter (Kim et al., 1990). Promoter sequence and deletion studies implicated two AP-1 binding sites as required for autoregulation, suggesting a role for the Fos–Jun complex. Footprinting studies confirmed binding at these sites by a purified Fos–Jun complex. The functional significance was studied by use of antisense *fos* and *jun* RNA expressing plasmids (Kim et al., 1990) using the antisense *jun* construct of Robert Chiu (Fig. 4) while the antisense *fos* construct utilized the fragment of Holt et al. (1986) in conjunction with a 1.6-kb mouse metallothionein promoter. In experiments similar to those for the analysis of autoregulation, A-549 human lung adenocarcinoma cells were cotransfected with a plasmid containing a TGF-β1 promoter–CAT construct together with antisense c-*fos* or c-*jun* or control plasmid DNA. In the presence of either antisense RNA expressing construct, TGF-β1-stimulated CAT synthesis was inhibited by over 80%. It was concluded that the AP-1 binding site mediated the autoregulation of TGF-β1 and that expression of both Jun and Fos components of the AP-1 complex is required.

Control of Chromosomal Aberrations

Chromosomal instability is a characteristic of tumor cells that may be important in the multistep progress of tumor progression (Fearon and Vogelstein, 1990). van den Berg et al. (this volume) have explored the role of FOS in this process and provide an additional potential effect of overexpression of c-*fos* in certain human tumors (Wu et al., 1990).

PROSPECTS AND APPLICATIONS

Antitumor therapy is an attractive potential of the antisense technique. In the case of c-*fos* and c-*jun* a number of concerns are apparent. Many of the results described here, especially for the various protooncogenes, support the emerging view that c-*fos* and c-*jun* are points of convergence of signal transduction pathways, so these genes may play

fundamental roles in many normal cells (Vogt and Bos, 1989; Marx, 1989). For example, c-*fos* may be essential for normal cell division in fibroblasts. Thus the use of antisense *fos* and *jun* RNA may lead to detrimental effects in normal cells and may be a poor use of the potential specificity of the antisense approach. Further, the role of c-*fos* in transformed cells is unclear. Although overexpression of c-*fos* causes chondroosseous tumors in transgenic animals, overexpression is rarely observed in human tumors, even in tumor types that are closely related to v- and c-*fos*-promoted murine tumors (Wu et al., 1990). We examined the expression of Fos protein in 30 human osteosarcomas using a variety of specific antisera at five concentrations and measured the extent of immunoreactivity, either by microdensitometry or by means of a panel of three trained pathologists (Wu et al., 1990). Nonlinear least-squares refinement of the data according to a model for immunotitration was used to show that 61% of the cases overexpressed Fos. However, the average increase in Fos expression was 1.5 times that of normal tissues or benign lesions (Wu et al., 1990). Similarly, in the case of EJ Ha-*ras*-dependent transformation of NIH-3T3 cells, a process that is believed to required activation of c-*fos*, increased c-*fos* transcription was not observed. Indeed in the EJ Ha-*ras*-transformed cells the ability of added growth factors to induce c-*fos* transcription was reduced—as for several other genes of *ras*-transformed cells (Ledwith et al., 1990). In the case of *sis*-transformed fibroblasts, c-*fos* was elevated in several transfected and G418-resistant control clones (Mercola et al., 1988). However, S. Edwards (personal communication) examined c-*fos* expression in over 20 subclones of the parental SSV-3T3 cells and observed that, while v-*sis* transcription was high and constant, c-*fos* expression was only slightly increased in a minority of subclones. Similarly, Fahrer et al. (1989) observed that, while c-*sis* and c-*fos* were commonly expressed in a series of 12 human sarcoma cell lines or biopsies, there was little correlation. On the other hand, it may be that the relevant level of Fos depends critically on tissue susceptibility. For example, E. Wagner and co-workers (E. Wagner, personal communication) have prepared a transgenic mice with exogenous c-*fos* expression determined by an H2 promoter and have prepared chimeric mice derived from embryonal carcinoma stem cells bearing human MT-c-*fos* constructs, all with 3′ viral LTRs. The tissue distribution of Fos expression was different from that of previous studies (Rüther et al., 1989) but again only chondroosseous lesions were observed—thus emphasizing the susceptibility of certain precursor cells to the effects of Fos expression.

A final caveat derives from the lack of any demonstration that complete tumor suppression can be achieved by use of antisense *fos* or *jun* RNA. Even for the two reported cases of reduced tumor growth (Mercola et al., 1988; Ledwith et al., 1990) antisense RNA expression and other mechanistic feature of an antisense RNA-dependent effect were not assessed. Thus a demonstration of the efficacy of antisense *fos* or *jun* RNA in suppression of solid tumor growth in vivo and determination of the consequences of exposing normal tissue to efficacious treatment levels would be very valuable.

Many of the studies summarized here provide evidence for the efficacy of antisense RNA as an analytical tool. This approach may be of use in determining the role of c-*fos*, c-*jun*, and other gene products in transformed cells including human tumors via analyses of subcultures. An appealing extension of this approach is the use of antisense DNAs to study functional differences between members of the superfamilies. Indeed, as noted, antisense DNAs with sequences complementary to individual Fos and Jun family members may be useful in differentiating among the roles of family members (Morris et al., 1991; Reysch and Schlingensiepen, 1991). Thus it will be of considerable interest to determine whether transformed cells exhibit particular specificities that can be targeted separately from universal or essential signal transduction pathways. Recent studies have clarified the nature of the acquisition of activated oncogenes and altered suppressor genes in several carcino-

mas (Fearon and Vogelstein, 1990). For a given tumor type, the acquisition in time of a finite number of recurring genetic alterations, but in varying combinations depending on the particular tumor, appears to be a reasonable expectation. Thus by the use of a series of antisense RNA expressing plasmids and/or DNAs, it may be possible to evaluate the significance of each candidate gene on phenotype of tumor cells in tissue culture. Further, as the targets of suppressor gene regulation become known, it may be possible to apply antisense RNA to these targets and further evaluate—and reverse—the effects of loss of suppressor regulation. Recently one such target for the retinoblastoma (suppressor) gene has been suggested to be *c-fos* itself (Robbins et al. 1990). Such information may be of value in defining the consequences of genetic alterations, in subtyping tumors, and in defining a minimum list of target genes for a rational therapy. Thus by using a combination of antisense agents and focusing on the particular "profile" of a particular tumor type, it may be possible to use lower doses of antisense agents that are less detrimental to normal processes. Recent advances in targeting double-stranded DNA and devising forms of DNA stable to enzymatic degradation and increased efficacy against a variety of viruses further suggest a broadening range of applications (Pine, 1990; J. Cohen, personal communication). Rossi and colleagues have developed a promising new approach based on the first application of ribozyme technology to *fos* transcripts (Scanlon et al., 1989). Ribozymes (synthetic hammerhead) were constructed to specifically bind to the fos mRNA at a 24 nt stretch that contained a ribozyme-susceptible GUC sequence. Addition of $MgCl_2$ led to specific cleavage. Complete digestion by the *fos* ribozyme required the presence of 3 m*M* $MgCl_2$. Thus ribozymes may be a useful adjunct.

There appear, therefore, to be a variety of analytical, diagnostic, and potential therapeutic approaches that may be of value in understanding and intervening in the expression of the transformed phenotype by antisense RNA methods.

SUMMARY

The use of antisense *fos* and *jun* RNA has contributed to our understanding of cell-cycle regulation, differentiation, gene regulation, and, in particular, transformation. In all effective cases, expression of antisense *fos* RNA via stable transfection of plasmids has led to a reduction of steady-state *c-fos* transcript levels implying a mechanism of action that involves the breakdown of RNA involved in RNA duplex formation. A wide range of sequences all containing 5′ portions of the *c-fos* gene have been used as a source of antisense RNA production or DNA antisense oligonucleotide synthesis. Similarly the 5′ coding region of *c-jun* has been used for the preparation of plasmids designed to express antisense RNA. Induction of antisense *c-fos* RNA in fibroblasts that have been stimulated to divide by platlet-derived growth factor leads to inhibition of the characteristic transient expression of *c-fos* and cell division illustrating the essential role of *c-fos* expression for the reentry of quiescent fibroblasts into the cell cycle. However at least one other cell type, mouse F9 embryonal carcinoma cells, do not require *c-fos* for growth.

The introduction of plasmids designed to express antisense *fos* or *jun* RNA into fibroblasts relieves repression of the *c-fos* promoter thus providing confirmatory evidence that the *c-fos* product acts with the *c-jun* product as a repressor of its own promoter. Similar transient transfection studies have been carried with both Fos expression vectors and cotransfected chimeric promoter–chloramphenicol transferase (CAT) constructs in order to study the role of Fos and Jun products in regulation. In a variation of this approach stably transfected fibroblast lines that conditionally express c-Ha-*ras*, *c-mos*, or *c-src* were inhibited in their ability to conditionally activate a collagen promoter-CAT construct but cotransfection of a antisense *fos* RNA expressing plasmid blocked activation thus suggesting that *c-fos* activity is required for regulation by these protooncogenes. Expression of antisense *fos* RNA in stably transfected fibroblasts lines transformed by

c-Ha-*ras* or *v-sis* demonstrates that *c-fos* mediated many of the manifestations of the transformed phenotype in *ras* and *sis* transformed cells including tumor formation and growth rate. Recent preliminary studies using synthetic single-stranded oligonucleotide DNA complementary to the *fos* or *jun* family members suggest that *c-jun* expression is required for growth of a human mammary carcinoma cell but had little effect on murine fibroblasts whereas antisense complementary to *jun*-B or *jun*-D led to markedly increased growth in both cases. Differential effects of the Fos and Jun family members have been reported in the control of the interleukin-2 (IL-2) gene of thymoma and T cells. Antisense DNA complementary to *jun*-B or *fos*-B inhibited activation while antisense *jun*-D or *c-fos* had no effect on the expression of the IL-2 construct. The results suggest that for *fos* and *jun* expression the various family members may have differential roles that can be discriminated by antisense techniques. *Fos* ribozymes have been prepared and shown to cleave *fos* RNA in vitro. Confirmatory studies including a demonstration that antisense RNA and DNA inhibit target gene expressing are required. The application of antisense *fos* and *jun* RNA and DNA for therapy was discussed.

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Expression of Antisense RNA against Initiation Factor eIF-4E mRNA in HeLa Cells Results in Lengthened Cell Division Times, Diminished Translation Rates, and Reduced Levels of Both eIF-4E and the p220 Component of eIF-4F

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HeLa cells were transformed to express antisense RNA against initiation factor eIF-4E mRNA from an inducible promoter. In the absence of inducer, these cells (AS cells) were morphologically similar to control cells but grew four- to sevenfold more slowly. Induction of antisense RNA production was lethal. Both eIF-4E mRNA and protein levels were reduced in proportion to the degree of antisense RNA expression, as were the rates of protein synthesis in vivo and in vitro. Polysomes were disaggregated with a concomitant increase in ribosomal subunits. Translation in vitro was restored by addition of the initiation factor complex eIF-4F but not by eIF-4E alone. Immunological analysis revealed that the p220 component of eIF-4F was decreased in extracts of AS cells and undetectable in AS cells treated with inducer, suggesting that p220 and eIF-4E levels are coordinately regulated. eIF-4A, another component of eIF-4F, was unaltered.

Formation of the 48S initiation complex is an ATP-dependent process whereby mRNA becomes bound to the 43S initiation complex (3, 61). This is considered to be the rate-limiting step in protein synthesis under normal (e.g., non-virus-infected) conditions, a conclusion that is based on the observations that (i) 48S complexes are considerably less abundant than 43S complexes (11) and can usually be detected only in the presence of inhibitors (56) and (ii) the ATP-dependent step of initiation is rate limiting (42). The polypeptides which are most directly involved in binding of mRNA to the 43S complex belong to the eIF-4 group of initiation factors (reviewed in references 49 and 58): eIF-4A, a 46-kDa RNA-dependent ATPase; eIF-4B, an 80-kDa polypeptide which stimulates the activity of eIF-4A; eIF-4E, a 25-kDa cap-binding protein; and a 220-kDa component referred to as either p220 (23) or eIF-4Fγ (55). Collectively, these factors carry out the ATP-dependent unwinding of secondary structure in mRNA beginning from the 5′ terminus, which accompanies (or precedes) the migration of the 40S ribosomal subunit to the initiation codon.

eIF-4E is a phosphoprotein and exists as a mixture of phosphorylated and unphosphorylated forms in rabbit reticulocytes (51) and HeLa cells (8, 16). The overall rate of protein synthesis is correlated with the degree of eIF-4E phosphorylation in a number of systems: both are decreased in HeLa cells following heat shock (16) and during mitosis (5), and both are increased in reticulocytes stimulated with phorbol esters (47), 3T3 L1 or HIR 3.5 cells stimulated with insulin (41, 48), 3T3 fibroblasts stimulated with serum (34), B lymphocytes stimulated with bacterial lipopolysaccharide or phorbol esters (53), and epithelial cells stimulated with epidermal growth factor (15). Furthermore, whereas eIF-4E accompanies the transfer of mRNA to the 48S initiation complex (29), a variant containing Ala rather than Ser at the major phosphorylation site (designated the [Ala^{53}]eIF-4E variant) fails to be transferred (33). These observations suggest that phosphorylation of eIF-4E is obligatory for its action in the transfer of mRNA to the 43S initiation complex.

More direct evidence that eIF-4E plays an important role in controlling the overall rate of protein synthesis comes from cell transfection studies with vectors expressing eIF-4E. Overexpression of eIF-4E by three- to eightfold over endogenous levels causes shortening of cell doubling times and loss of contact inhibition of rat fibroblasts (40) and HeLa cells (12). Transfection with vectors expressing the [Ala^{53}]eIF-4E variant does not cause these effects. These results, together with the knowledge that eIF-4E is the least abundant of the initiation factors (16) and is present at only 1/10 the molar concentration of mRNA (29), implicate eIF-4E as a major determinant of the overall rate of protein synthesis.

The role of p220 in initiation is less well defined. p220 can be isolated from high-salt-treated cell lysates or ribosomal pellets in complexes with eIF-4E alone (9, 22), with eIF-4A and eIF-4E (17, 26) (in this case the complex is termed eIF-4F), with eIF-4A, eIF-4B, and eIF-4E (26), and with eIF-3 (27). Whereas eIF-4E alone was originally reported to restore protein synthesis to extracts of poliovirus-infected cells (62), subsequent studies indicated that high-molecular-weight complexes containing p220 as well as eIF-4E were required (60). An explanation for this was provided by the finding that poliovirus infection causes proteolytic cleavage of p220 (23). Buckley and Ehrenfeld (9), on the other hand, could not demonstrate the existence of a p220:eIF-4E complex in the total cytoplasmic extract of HeLa cells not treated with high salt. Thus, while it is clear that p220 is intimately involved in the formation of 48S initiation complexes involving capped mRNA, neither its mechanism of action nor the nature of its interactions with other initiation factor polypeptides or with the 40S ribosome is fully understood.

In this study, we have further explored the role of eIF-4E by expressing antisense RNA (AS RNA) against eIF-4E mRNA. The results are consistent with the idea that eIF-4E

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plays a key role in determining the overall rate of protein synthesis and cellular growth. Unexpectedly, we also found that expression of AS RNA reduced the level of p220 as well as the level of eIF-4E.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The *Escherichia coli*-mammalian shuttle vector RDB, which replicates episomally, has been described previously (12, 13). RDB-0 is the vector alone, containing no inducible promoter or antisense sequences. Plasmid pG-eIF-4E is pGem-7Zf(+) (Promega Biotec) containing the human eIF-4E cDNA from plasmid pTCEEC (12).

All oligonucleotides were synthesized at the University of Kentucky Macromolecular Synthesis Facility. These are (i) antisense oligonucleotide 5′-AGTCGCCATCTTAGATCGAT-3′, complementary to nucleotides (nt) −11 to +9 of human eIF-4E mRNA (numbering system of Rychlik et al. [50]); (ii) polymerase chain reaction (PCR) 5′ primer 5′-TACACATCC CCAGAATCCATAAAT-3′, the same sense as nt 885 to 909 of eIF-4E mRNA; and (iii) PCR 3′ primer 5′-TAACCAA AGCAAAATAACCTAAGT-3′, complementary to nt 1520 to 1544 eIF-4E mRNA. Optimal annealing temperatures for PCR were calculated by using the computer program OLIGO (52). PCR conditions using these temperatures were then optimized to produce a single product.

The vector to express AS RNA against eIF-4E mRNA was constructed from RDB-DRE (13) by insertion of a double-stranded form of the antisense oligonucleotide into the *Xba*I site of the polylinker. *E. coli* JM110 cells were transformed, and plasmids from ampicillin-resistant colonies were screened for the presence of a *Cla*I site, which is created by the insertion of the antisense oligonucleotide. The fidelity of the construction and orientation of the insert were confirmed by DNA sequencing. The plasmid containing the antisense sequence in the proper orientation was called RDB-AS.

Cell culture, transfection protocols, and selection of G418-resistant clones were carried out as described previously (12). Where indicated, 10 nM tetrachlorodibenzo-*p*-dioxin (TCDD) was added to the culture medium.

Determination of RNA levels. RNA was isolated as follows. Cells were resuspended in *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES)-buffered saline containing 2 mM EDTA. After pelleting by centrifugation at 800 × *g*, they were resuspended in 0.5 ml of lysis buffer (50 mM Tris, 0.5% sodium dodecyl sulfate [SDS], 0.1 mg of proteinase K per ml; adjusted to pH 7.2) and incubated at 37°C for 6 h. The NaCl concentration was then adjusted to 1 M, and the samples were kept on ice overnight to precipitate the bulk of the chromosomal DNA. After a 5-min centrifugation, the supernatant was phenol extracted once, and nucleic acids were precipitated and washed with 70% ethanol. Total RNA was resuspended in 50 mM Tris–2.5 mM EDTA, pH 7.2. Integrity of the RNA was assessed by denaturing agarose gel electrophoresis. For the PCR quantitations, RNA was further treated with DNase (RQI; Promega), phenol-chloroform-isoamyl alcohol extracted, and ethanol precipitated. RNA concentrations were determined spectrophotometrically.

RNase protection assay. A 771-base, minus-sense probe was generated by in vitro transcription with SP6 polymerase of pG-eIF-4E linearized at the *Acc*I site. Transcription reaction mixes contained (in a total volume of 20 μl) 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol, 0.4 mM each ATP, CTP, and GTP, 0.2 mCi of [5,6-^{3}H]UTP, and 1 μg of pG-eIF-4E. Transcription reaction mixes were incubated 1 h at 37°C and then heated to 70°C for 5 min. DNase RQ1 (5 U) was added, and the samples were incubated at 30°C for 15 min. Samples were adjusted to 10 mM EDTA and 0.2 M NaCl and extracted with phenol-chloroform, and the RNA was precipitated with 3 volumes of ethanol. The precipitate was washed once with 70% ethanol and dried. The probe thus produced had a specific activity of 2.3×10^7 cpm/μg. Test RNA was annealed to the probe (2×10^5 cpm) overnight at 50°C in 50 μl of a solution containing 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.7), 0.4 M NaCl, 1 mM EDTA, and 80% formamide. Then 0.3 ml of an ice-cold solution containing 0.3 M NaCl, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 15 μg of RNase A (Boehringer) was added, and the solution was incubated at 30°C for 1 h. Following extraction of the sample with phenol-chloroform, 5 μg of carrier yeast tRNA was added, and nucleic acids were precipitated with 2 volumes of ethanol.

PCR amplification. One to five micrograms of total RNA was reverse transcribed as described by Wang et al. (64), with minor modifications. A 10-μl reverse transcription reaction mix contained 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, 1 mM dithiothreitol, 50 μM each deoxynucleoside triphosphate, 5 to 10 U of RNasin (RNA Guard; Promega), 0.1 μg of the 3′ primer, and 3 U of avian myeloblastosis virus reverse transcriptase (Promega). Prior to the addition of the other components, the RNA and primer were heated at 65°C for 10 min in diethylpyrocarbonate-treated H_2O. Reactions were carried out at 37°C for 1 h. Aliquots of the reverse transcriptase reaction were combined with carrier rRNA to produce a total of 0.05 to 0.4 μg of total RNA and were amplified in a 100-μl reaction mix containing 2 U of *Taq* DNA polymerase (AmpliTaq; Perkin-Elmer-Cetus Corp.), 0.3 μg of each primer, 200 μM deoxynucleoside triphosphates, and 1 to 10 μCi of [α-^{32}P]dATP. The PCR reaction was carried out in a Perkin-Elmer-Cetus thermocycler at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Aliquots of 1 to 3 μl were taken at selected points by pausing the machine for 1 min following the 72°C step, spotted onto Whatman 3MM filter disks, and batch washed in trichloroacetic acid (TCA); the radioactivity was determined in toluene-based scintillation fluid.

Northern (RNA) blots. Actin mRNA was detected in total RNA by Northern analysis (13), using human β-actin cDNA in pGEM3 (1.95×10^8 cpm/μg) as the probe. AS RNA was similarly detected with BS-DRE (13) (2.2×10^8 cpm/μg) as the probe. The relative abundances of actin mRNA and AS RNA were calculated from the specific activities of the two probes and the Northern results shown in Fig. 1B (inset) and Fig. 4C. The ratio of the AS RNA signal from AS RNA-expressing HeLa cells (AS cells) in the absence of inducer to that of actin mRNA was 0.022, whereas that from the cells plus inducer was 0.061.

Immunologic analysis. Extracts from HeLa and AS cells were prepared as described previously (12). The protein concentration in each sample was determined by the method of Bradford (6), and equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using an 8% gel. The proteins were transferred to a polyvinylidene difluoride membrane (MSI Nitroplus 2000; Micron Separations Inc., Westboro, Mass.) and probed with a monoclonal antibody against p220 (19; gift from Diane Etchison) or eIF-4A (17; gift from Hans Trachsel) as described by Winston (66). The secondary antibody was affinity-purified

horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, Calif.). The antigen-antibody complex was visualized by using the chromogenic substrate 3,3'-diaminobenzidine hydrochloride.

Measurement of protein synthesis. In vivo protein synthesis rates were measured by incorporating [3,4,5-^{3}H]leucine at 30 μCi/ml into cells for 3 h. A cytoplasmic extract was prepared and subjected to either SDS-PAGE or TCA precipitation. The specific radioactivity of the leucine pool was determined by extracting the TCA-soluble fraction with 5 volumes of ether and subjecting equal aliquots to scintillation spectrometry and automated amino acid analysis in the University of Kentucky Macromolecular Structure Analysis Facility.

In vitro protein synthesis was performed as follows. Cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The pelleted cells were homogenized in 2.5 volumes of hypotonic buffer containing 20 mM HEPES (pH 7.4), 10 mM NaCl, 1.5 mM magnesium acetate, 0.3% Brij 58, 300 U of RNasin per ml, 2 μg of leupeptin and pepstatin per ml, and 50 μM hemin. Cytoplasmic extracts were obtained by sedimenting nuclei and cell debris at 30,000 × *g*. Protein synthesis was carried out at 30°C for 1 h in 50-μl reaction mixes containing 30 μl of cell extract, 10 μl of reaction mix (0.15 M NaCl, 0.2 M potassium acetate, 5 mM dithiothreitol, 74 mM creatine phosphate, 40 U of creatine phosphokinase per ml, 1 mM ATP, 1 mM GTP, 0.1 M HEPES, pH 7.4), 35 μM amino acids minus leucine, and 50 μCi of [3,4,5-^{3}H]leucine (NEN).

Polysome analysis. The protocol of White et al. (65) was carried out with the following modifications: (i) the cells were detached by using a solution of 0.05% (wt/vol) trypsin and 0.53 mM EDTA and washed with PBS, (ii) no NaCl was added during purification of the cytoplasmic extracts, and (iii) the cytoplasmic extracts were layered onto linear 4.5-ml sucrose gradients (0.5 to 1.5 M), which were centrifuged for 50 min at 4°C in a SW60Ti rotor at 50,000 rpm.

Protein preparations. eIF-4E was purified from human erythrocytes as described by Rychlik et al. (51) and from HeLa cells as described by De Benedetti and Rhoads (12). eIF-4F was purified from rabbit reticulocyte ribosomal salt wash by m^{7}GTP-Sepharose chromatography followed by Mono Q chromatography as described by Lamphear and Panniers (39).

RESULTS

Phenotype of transfected cells. The vector used to express AS RNA was derived from BK virus and pSV-2neo and contained a mouse mammary tumor virus (MMTV) long terminal repeat promoter linked to a dioxin-responsive enhancer (13). A sequence complementary to 20 nt near the 5' terminus of eIF-4E mRNA was placed under control of the inducible promoter. Cells harboring this vector are resistant to the antibiotic G418 as a result of the expression of the aminoglycoside phosphotransferase gene present in the construct. Cells transformed with this vector (hereafter referred to as AS cells) grew slowly, with a doubling time of about 100 h (Fig. 1A), whereas untransfected HeLa cells doubled in approximately 25 h (Fig. 1B). The fact that inhibitory effects were observed in the absence of inducer is consistent with previous observations that a low level of constitutive gene expression occurs with this promoter-enhancer combination (12, 13, 32). Addition of the inducer TCDD to AS cells caused further slowing of the growth rate and then a decline in cell number after 2 days (Fig. 1A). TCDD had no detectable effect on untransfected HeLa cells (Fig. 1B) or on cells transformed with RDB-0 (data not shown).

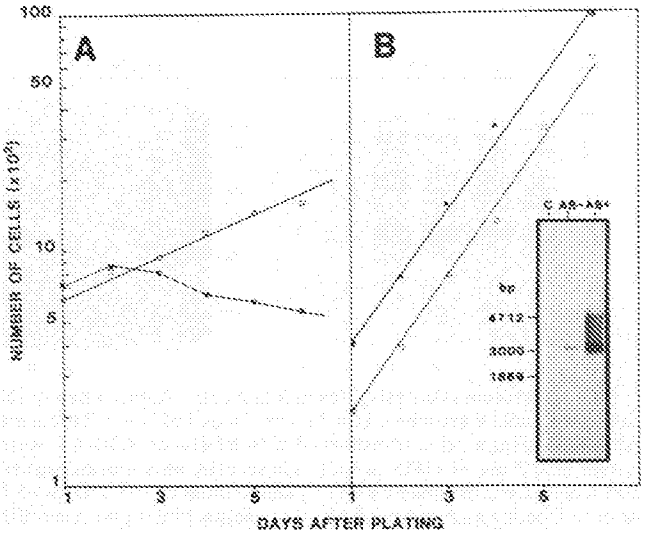

FIG. 1. Growth curves of HeLa and AS cells. Cells were plated in 25-cm^2 flasks with a graduated bottom. The average cell number in four random 1-cm^2 grids was taken each day, beginning 1 day after plating. (A) AS cells cultured in 0.2 mg of G418 per ml with (closed squares) and without (open squares) the inducer TCDD. (B) Control untransfected HeLa cells grown with (open circles) and without (closed circles) TCDD. (Inset) Northern analysis of antisense RNA produced in control HeLa cells (C), AS cells without inducer (AS−), and AS cells with inducer for 18 h (AS+). Total RNA was probed with BS-DRE (see Materials and Methods). The film was exposed for 21 days.

The copy number of this vector can be altered by changing the concentration of G418 in the culture medium (13). The 100-h doubling time in Fig. 1A was obtained with G418 at 0.2 mg/ml. When cells were cultured in G418 at 0.4 or 0.6 mg/ml, the doubling time increased to 170 h (data not shown). Conversely, when AS cells were maintained without G418 selection, they resumed normal growth rates in about 2 weeks, presumably because of a reduction of vector copies. HeLa cells transformed with RDB-0 grew at normal rates in the presence of G418 at either 0.2 or 0.4 mg/ml (data not shown). These results indicate that the phenotype of slow growth is due to the expression of eIF-4E antisense sequences and not to the vector per se, G418 or TCDD.

The nature of the RNA product containing the AS sequence was examined by Northern hybridization (Fig. 1B, inset). The predominant product was approximately 3 kb, consistent with the RNA beginning with the MMTV promoter and ending with the termination signal and poly(A) addition site of BK virus. The RNA downstream of the AS sequence is derived from Bluescript. Control HeLa cells contained no cross-hybridizing RNAs (lane C). The major AS RNA was present at 2.2% the molar concentration of β-actin mRNA in uninduced cells (lane AS−), and this increased to 6.1% after 18 h of induction (lane AS+).

In vivo protein synthesis. If AS RNA decreases the intracellular levels of eIF-4E, and if eIF-4E is rate limiting for protein synthesis, then the slow growth of AS cells could be due to a reduction in overall protein synthesis rate. To test this, we measured the incorporation of [^{3}H]leucine into protein for 6 h in control and AS cells, the latter grown in G418 at 0.6 mg/ml. The rate of leucine incorporation was

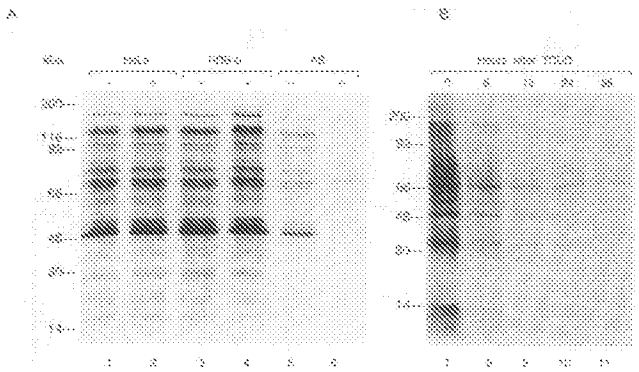

FIG. 2. Protein synthesis rates in intact cells. Approximately 10^6 cells per sample were labeled for 3 h with 30 μCi of [3,4,5-^{3}H]leucine per ml. (A) HeLa cells, transformed with RDB-0 or RDB-AS, were grown in 0.2 mg of G418 per ml. These cells plus untransformed HeLa cells were incubated with (+) and without (−) TCDD for 48 h prior to labeling and lysed in 0.15 ml of sample buffer (38). One-fifth of the sample was subjected to SDS-PAGE on 10% gels. (B) AS cells were labeled with [3,4,5-^{3}H]leucine for 3 h at the times indicated following the addition of TCDD. The positions of molecular weight standards are indicated. The TCA-precipitable radioactivities of selected samples were 26,285 (lane 3), 9,385 (lane 5), 2,165 (lane 6), 10,775 (lane 7), 7,040 (lane 8), 5,985 (lane 9), 3730 (lane 10), and 2,980 (lane 11) cpm/μl. The film for panel B was exposed three times longer than that for panel A.

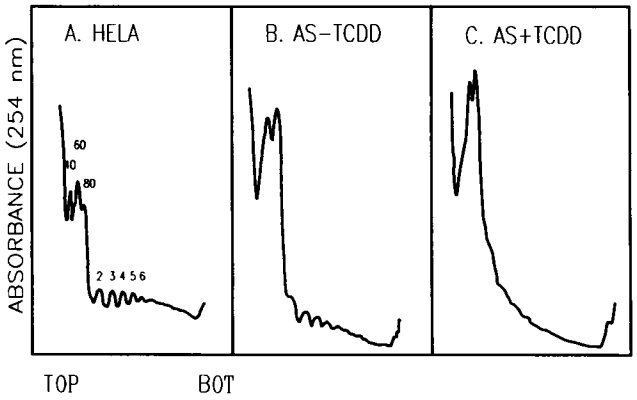

FIG. 3. Distribution of polysomes after AS RNA expression. Untransfected HeLa cells (A) and AS cells maintained in 0.2 mg of G418 per ml without (B) or with (C) treatment for 42 h with TCDD were harvested and analyzed for polysomes as described in Materials and Methods. Ribosomal subunits and monosomes are indicated by 40, 60, and 80. Disomes, trisomes, etc., are indicated by 2, 3, etc. The direction of sedimentation in each case was left to right.

reduced by 10-fold in AS cells (10,325 versus 1,230 cpm/10^4 cells). Addition of TCDD at the beginning of the 6-h labeling period did not change the rate of incorporation in either control or AS cells, suggesting that the inducer requires a period of time to produce its effect.

In a second experiment, AS and control RDB-0 cells were maintained in 0.2 mg of G418 per ml to produce a lower copy number of the vector. The transfected cells as well as untransfected HeLa cells were then incubated with and without TCDD, in this case 48 h prior to labeling with [^{3}H]leucine for 3 h (Fig. 2). The leucine pool-specific radioactivities of the various cell lines, with or without TCDD treatment, were the same to within 6%. Thus, the intensity of bands in Fig. 2 is proportional to the protein synthesis rate. The overall rate of protein synthesis was reduced 2.8-fold in the AS cells (Fig. 2A, lane 5 versus lane 3), and the addition of TCDD caused a further 4.3-fold decrease (lane 6). Protein synthesis was the same in HeLa and RDB-0 cells, and TCDD had no effect (lanes 1 to 4). Synthesis of most proteins was reduced in AS cells, but that of some proteins was more resistant to the general inhibition. These presumably result from the translation of "strong" mRNAs, i.e., those having the least requirement for eIF-4 group initiation factors (reviewed in reference 41).

We also studied the time course of TCDD action on the rate of protein synthesis in AS cells. The addition of TCDD produced a progressive decrease in protein synthesis rates over a 36-h period (Fig. 2B). Taken together, these experiments indicate that the expression of eIF-4E AS RNA, whether determined by vector copy number or by induction of the promoter, reduces the in vivo rate of protein synthesis in a dose- and time-dependent manner.

If the depletion of eIF-4E is responsible for the observed inhibition of protein synthesis, one would expect polysomes to be disaggregated because eIF-4E acts at the step wherein mRNA becomes bound to the ribosome. To test this prediction, polysome profiles were measured in control HeLa cells and AS cells in the presence and absence of TCDD (Fig. 3). The results indicated that polysomes were decreased in AS cells with a corresponding increase in ribosomal subunits (Fig. 3B versus 3A), and when TCDD was added to the medium, polysomes became undetectable (Fig. 3C).

eIF-4E mRNA levels. AS RNA is believed to exert its effect on target gene expression by inhibiting the synthesis, processing, transport, or translation of a specific mRNA (24). To distinguish between translational and nontranslational effects in the case of eIF-4E AS RNA, we measured the level of eIF-4E mRNA. Cells were treated with and without TCDD for 36 h, a period which does not result in cell death or even in the complete arrest of cell growth (Fig. 1A), and the relative levels of eIF-4E mRNA were determined by two independent methods. In the first, an RNase protection assay, a minus-sense probe was generated by in vitro transcription of the cDNA for eIF-4E with SP6 RNA polymerase (Fig. 4A; Fig. 4B, lane 1; note that the markers refer to mobility of double-stranded DNA and do not apply to the single-stranded RNA probe). RNase A completely digested the probe in the absence of complementary mRNA (lane 2). Hybridization of the probe to a full-length, plus-sense transcript, produced by transcription of the same plasmid with T7 RNA polymerase, resulted in a protected fragment migrating at 725 bp (lane 3), the expected position of the duplex RNA. Total RNA extracted from control cells yielded a strong band (lane 4). AS cells, by contrast, yielded considerably less of the protected band (lane 5), and TCDD treatment further reduced this level (lane 6). Bands corresponding to the protected probe were excised, and the radioactivity was determined by scintillation spectrometry (Fig. 4B, bottom). From the radioactivity in lanes 3 and 4, we calculate that eIF-4E mRNA represents 0.04% of total HeLa cell mRNA (see figure legend). AS cells and AS cells treated with TCDD contained 3- and 11-fold, respectively, less eIF-4E mRNA than did control cells. As a control that all mRNA species were not decreased by AS RNA expression, we also measured actin mRNA content during the course of TCDD induction (Fig. 4C). Actin mRNA levels did not change significantly over the 48-h period tested.

The second method used for mRNA quantitation was

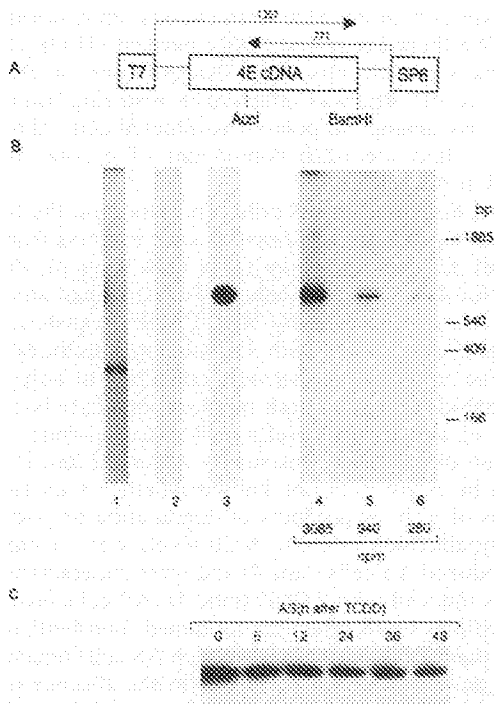

FIG. 4. Quantitation of eIF-4E mRNA by RNase protection. (A) Transcription template used to synthesize the eIF-4E mRNA plus- and minus-sense RNAs. (B) RNase protection assays carried out as described in Materials and Methods. After ethanol precipitation of the protected RNA, the samples were resuspended in 10 mM Tris-HCl (pH 7.2)–1 mM EDTA–15% glycerol and separated on a nondenaturing 1.5% agarose gel, using *Dde*I-cut pGem-7Zf(+) DNA as markers. In this system, RNA duplexes shorter than 1.5 kb comigrate with DNA duplexes of the same size. Lane 1, 500 cpm of single-stranded probe (synthesized with SP6 polymerase); lane 2, probe digested with RNase A in the absence of complementary RNA; lane 3, signal obtained after annealing of probe to 5 ng of plus-sense RNA (synthesized with T7 polymerase). Total RNA (70 μg) from control untransfected cells (lane 4), AS cells grown in 0.2 mg of G418 per ml (lane 5), and AS cells treated for 48 h with TCDD (lane 6) was annealed to the probe and digested with RNase A. The radioactivity in the major band of selected lanes is shown at the bottom. The level of eIF-4E mRNA in control cells was estimated as follows. Five nanograms of the T7 transcript protected 27,930 cpm of the probe. Assuming that 2% of total RNA is mRNA, the 70 μg of total RNA used in protection assays contained 1,400 ng of total mRNA, and this protected 3,085 cpm of probe. 3,085/27,930 × 5 ng = 0.55 ng of eIF-4E mRNA. 0.55/1,400 = 0.0004. Thus, eIF-4E mRNA represents 0.04% of total HeLa mRNA. (C) Northern analysis of actin mRNA. Total RNA was extracted from AS cells at various times after addition of TCDD as indicated and analyzed by Northern blotting using a β-actin cDNA probe (see Materials and Methods). The film was exposed for 1 day.

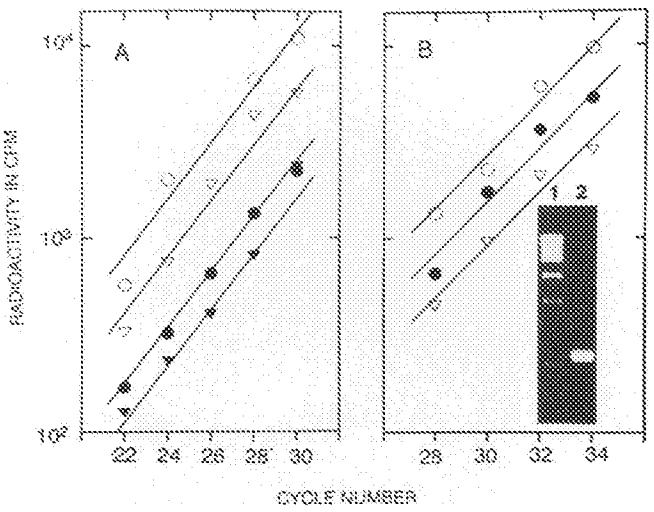

FIG. 5. Quantitation of eIF-4E mRNA in control and AS cells by PCR. (A) Dose response quantitation of eIF-4E mRNA in total RNA isolated from continuous rat embryo fibroblast cells. The amounts of total RNA used for PCR were 0.4 μg (open circles), 0.2 μg (open triangles), 0.1 μg (closed circles), and 0.05 μg (closed triangles). Carrier RNA was added after reverse transcription, and the samples were subjected to PCR amplification in the presence of [α-^{32}P]dATP, using eIF-4E mRNA-specific primers. Products were quantitated by TCA precipitation and scintillation spectrometry. (B) Effect of AS RNA on eIF-4E mRNA levels. Total RNA (0.1 μg) from untransfected HeLa cells (open circles), AS cells (closed circles), and AS cells treated with TCDD as for Fig. 3 (open triangles) was analyzed as for panel A. (Inset) Ethidium bromide-stained 2.5% agarose gel of *Hin*dIII-cut λ DNA (lane 1) and the eIF-4E mRNA-specific PCR product (lane 2).

selective reverse transcription of eIF-4E mRNA followed by PCR in the presence of [α-^{32}P]dATP. This yielded a single PCR product of 659 bp which could be quantitated directly by precipitation with TCA and scintillation spectrometry (Fig. 5B, inset). To demonstrate that the method was quantitative, we subjected several dilutions of reverse-transcribed RNA from a single source to PCR (Fig. 5A). The product increased exponentially for each dilution and was proportional to the amount of RNA added, provided that it was measured in the exponential range. AS cells contained 1.7-fold less eIF-4E mRNA than did control HeLa cells by this assay, and AS cells treated with TCDD for 36 h contained 3-fold less than did control cells (Fig. 5B). The quantitative discrepancy between the two methods is not understood, but it is clear that eIF-4E mRNA was significantly reduced in AS cells and that a further decrease occurred upon induction of the promoter with TCDD.

eIF-4E protein levels. The level of the eIF-4E protein was measured in cytoplasmic extracts from control HeLa and AS cells grown in 0.6 mg of G418 per ml with or without a 48-h induction with TCDD. eIF-4E was affinity purified on m^{7}GTP-Sepharose columns, and aliquots of both total protein and column-bound protein were separated by SDS-PAGE and stained with Coomassie blue (Fig. 6). Total protein patterns were similar (lanes 1 to 3), but eIF-4E was detectable only in the column-bound fraction of control cells (lane 4 versus lanes 5 and 6). In a similar experiment, the vector copy number was decreased by growing the cells in 0.2 mg of G418 per ml in an attempt to obtain a less severe reduction of eIF-4E. Under these conditions, AS cells contained sevenfold less eIF-4E than did control cells, as estimated by densitometry, whereas eIF-4E could not be detected in TCDD-treated AS cells (data not shown). Interestingly, an m^{7}GTP-Sepharose-enriched protein of 220 kDa was also decreased in AS compared with control cell extracts (lane 4 versus lanes 5 and 6). On the basis of its molecular weight and retention on m^{7}GTP-Sepharose, this is likely to be the p220 component of eIF-4F (17, 26, 60).

In vitro protein synthesis. The results presented thus far indicate that AS cells grow slowly, that protein synthesis in vivo is decreased up to 10-fold, and that the cellular levels of both eIF-4E mRNA and eIF-4E protein are decreased in

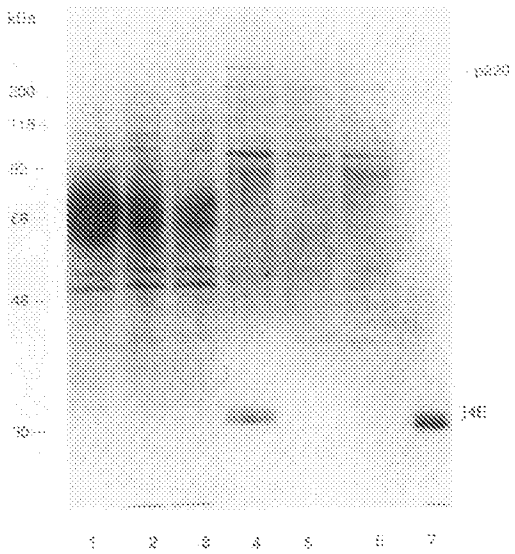

FIG. 6. Quantitation of eIF-4E in control and AS cells. The cytoplasmic extract from 5×10^7 cells (3 ml) was applied to a 2-ml m^7GTP-Sepharose column, and the bound protein was eluted with m^7GTP and precipitated with TCA (12). The protein was resuspended in Laemmli sample buffer, subjected to SDS-PAGE, and stained with Coomassie blue. The positions of molecular weight markers, eIF-4E, and a 220-kDa polypeptide, which is thought to be the p220 component of eIF-4F, are shown. AS cells were grown in the presence of 0.6 mg of G418 per ml, and the gel used was 10%. Lanes: 1 to 3, 30 μl of total protein from control HeLa cells, AS cells, and AS cells treated for 48 h with TCDD, respectively; 4 to 6, m^7GTP-Sepharose-bound protein from the same extracts (the entire sample); 7, 1.7 μg of purified eIF-4E from human erythrocytes. eIF-4E migrates as doublet when incompletely reduced (51). The dark bands at around 66 kDa in lanes 1 and 2 are bovine serum albumin, which was not completely removed when the cells were collected.

proportion to the expression of AS RNA. The most straightforward interpretation of these findings is that cell growth is slowed because of a decrease in protein synthesis resulting from the loss of eIF-4E. If so, then extracts of AS cells should likewise be restricted in protein synthetic capacity, and this should be restored with exogenous eIF-4E. Cell extracts were prepared from control and AS cells and assayed for [3,4,5-^{3}H]leucine incorporation. As observed for intact cells (Fig. 2), protein synthesis in extracts of AS cells was drastically reduced (Fig. 7A, lane 2 versus lane 1). Surprisingly, however, the addition of purified eIF-4E did not stimulate translation to any significant degree (Fig. 7B, lanes 8 to 12; note that the aliquots analyzed in lanes 8 to 12 were three times larger than that in lane 7). The highest level of eIF-4E added (lane 8) corresponded to fivefold more than was present endogenously in the cell-free system from control cells (see figure legend). It was unlikely that the lack of stimulation was due to loss of activity of eIF-4E itself, since eIF-4E purified by m^7GTP-Sepharose retains its activities of cap binding, association with the 48S initiation complex (33), and restoration of translation in m^7GTP-inhibited lysates (28).

The failure of eIF-4E to stimulate translation was unexpected, since the AS RNA was targeted specifically to eIF-4E mRNA. However, this is reminiscent of the inability of eIF-4E to restore translation to extracts of poliovirus-infected cells (60). In the latter case, high-molecular-weight complexes containing p220 were effective in restoring translation. We therefore tested highly purified eIF-4F (Fig. 7C) on extracts of AS cells (Fig. 7A). Addition of eIF-4F, in contrast to eIF-4E, was effective in restoring translational activity. By analogy to poliovirus-infected cells, this finding suggested that the p220 component of eIF-4F might be affected in AS cells.

p220 is decreased in AS cells. To determine the levels of p220 directly, we prepared cytoplasmic extracts from HeLa cells and AS cells grown in G418 at 0.2 mg/ml, the latter treated for 48 h with and without TCDD. Equal amounts of protein were separated by SDS-PAGE, transferred to a membrane, and probed with a monoclonal antibody against p220. The antibody recognized a collection of polypeptides in the 200-kDa range in both rabbit reticulocyte lysate (Fig. 8, lane 6) and control HeLa cell extracts (lane 1). This collection of bands is consistently observed and is considered to be p220; it is not known whether they represent isoforms of p220 or products of degradation or posttranslational modification (19, 20). p220 levels were decreased in the uninduced AS cells (lane 4) and were undetectable in the AS cells induced with TCDD (lane 5). AS cells grown for 1 week without G418 selection contained intermediate levels of p220 (lane 2). As noted above, such AS cells regain almost normal growth rates after 2 weeks in the absence of G418. Addition of TCDD to these cells decreased the p220 level only slightly (lane 3). Presumably, the recovery of normal growth rates is due to the loss of vector, so that TCDD addition results in insufficient AS RNA to produce a major effect. We did not observe the breakdown products of p220 (100 to 130 kDa) which are characteristic of poliovirus-infected cell extracts (23), though the monoclonal antibody used is capable of recognizing such products (19).

Decay of eIF-4E, p220, and protein synthesis rates after induction with TCDD. To analyze the relationship between the levels of eIF-4E and p220 and the overall in vivo rate of protein synthesis, we determined all three parameters in a single experiment as a function of time after addition of TCDD (Fig. 9). AS cells grown in 0.2 mg of G418 per ml were treated with TCDD, and aliquots from multiwell flasks were removed at intervals. Protein synthesis was determined by pulse-labeling cells during the last 3 h of each time point. A parallel set of cells which had been previously labeled to equilibrium with [3,4,5-^{3}H]leucine was used for quantitation of eIF-4E levels. Another set was analyzed by Western immunoblotting to measure p220 levels. The results indicated that except for a slight initial lag, eIF-4E and p220 decayed with nearly the same kinetics. Protein synthesis decreased the most between 6 and 18 h, in parallel with eIF-4E and p220, and thereafter more slowly, presumably reflecting the residual translation of specific "strong" mRNAs.

Another component of the eIF-4F complex is eIF-4A, although the major portion of this factor exists in the free form (see the introduction). It was therefore of interest to determine whether eIF-4A levels were also decreased when AS RNA was expressed. eIF-4A was monitored immunologically during the course of TCDD induction (Fig. 9, double triangles). Interestingly, over the period in which eIF-4E and p220 decreased to undetectable levels, eIF-4A was unchanged. In a separate experiment, the level of eIF-4A in control HeLa cells was found to be the same as in AS cells (data not shown).

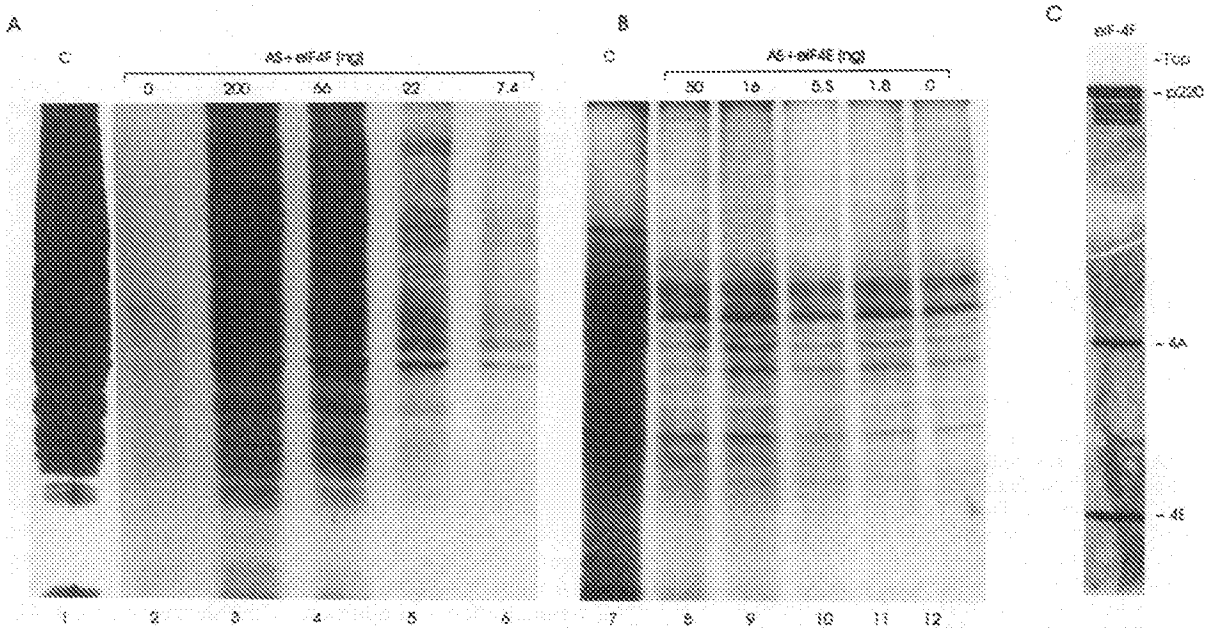

FIG. 7. Effect of added eIF-4F and eIF-4E on translation by extracts of AS cells. Proteins were labeled with [3,4,5-^{3}H]leucine in extracts of control HeLa cells (C; lanes 1 and 7) and AS cells grown in 0.6 mg/ml G418 (AS), separated by SDS-PAGE, and visualized by fluorography. (A) The indicated amounts of rabbit reticulocyte eIF-4F were added to 50-μl translation reactions. Aliquots of 5 μl were loaded on the gel. (B) Same as panel A except that human erythrocyte eIF-4E was added to translation reaction mixes. The aliquot loaded for control cell extracts (lane 7) was 5 μl, whereas that for AS extracts (lanes 8 to 12) was 15 μl. From the data in Fig. 6, we estimate that 1.0 μg of eIF-4E is present in 3 ml of HeLa extract. Thus, the control in vitro translation system (lane 7) contained 10 ng of endogenous eIF-4E in a 50-μl reaction mixture. (C) SDS-PAGE of the eIF-4F preparation used in panel A. Staining was with silver.

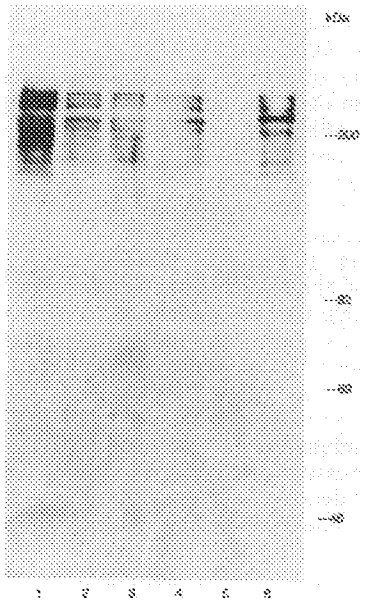

FIG. 8. Immunological analysis of p220 in control and AS cells. Cell extracts were prepared from HeLa and AS cells, and 150 μg of protein from each sample was analyzed by immunoblotting with an anti-p220 antibody. Lanes: 1, control HeLa cells; 2, AS cells cultured without G418 for 1 week; 3, same as lane 2 but treated with TCDD for 48 h; 4, AS cells cultured in 0.2 mg of G418 per ml; 5, same as lane 4 but treated with TCDD for 48 h; 6, 5 μl of rabbit reticulocyte lysate.

DISCUSSION

Determining the mechanisms which regulate the overall rate of protein synthesis is central to a general understanding of cellular metabolism. Nearly all of the translational control mechanisms identified to date occur at the initiation step, so it is logical to study the initiation factors as targets and mediators of translational regulation. Most or all of the initiation factors have now been isolated and characterized in vitro. However, the sheer complexity of protein synthesis, involving the coordinated action of over 200 proteins and 100 RNAs, requires that the roles of initiation factors be defined using in vivo systems as well. The favorable genetic approaches available in *Saccharomyces cerevisiae* have permitted several initiation factors to be studied in vivo. eIF-2β was shown to be essential to the cell and to play a role in initiation codon selection (14). Similarly, an intact eIF-4A gene is required for survival of the yeast cell (4). The eIF-4E gene is likewise essential to yeast cells (1) and, interestingly, is the same as a gene involved in control of cell division, *cdc-33* (7). The phenotype of *cdc-33* mutants resembles cells arrested at the G_0/G_1 boundary during amino acid starvation.

In higher eukaryotes, studies of initiation factors involving disruption of genes or their replacement with mutated derivatives are not presently feasible. Alternative approaches for studying these factors in vivo include (i) expressing additional copies of the initiation factor, either of the normal sequence or of a variant, by transfecting cells with expression vectors and (ii) reducing the endogenous levels of the initiation factor with antisense sequences. The first of these approaches has been used for eIF-2α, whereby the essential role of Ser-51 in regulating this factor's activity was demon-

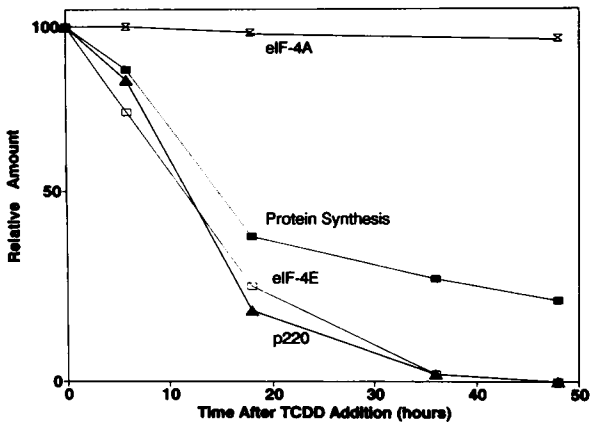

FIG. 9. Decay rates of eIF-4A, eIF-4E, p220, and protein synthesis in AS cells. AS cells (10^5) were incubated with TCDD in multiwell plates. At the times indicated, 15 μCi of [3,4,5-^{3}H]leucine per ml was added for 3 h. The cells were lysed in 0.5 ml of 1% SDS, proteins were precipitated with 10% TCA and collected on GFA filters, and the radioactivity was determined (closed squares). eIF-4E was measured in samples of 3×10^6 cells labeled to equilibrium with [3,4,5-^{3}H]leucine (50 μCi/ml) for 48 h. TCDD was added for the times indicated. The cells were lysed, and eIF-4E was isolated as described in Materials and Methods and separated on an SDS–10% polyacrylamide gel. After fluorography, the eIF-4E band was quantitated with a laser densitometer (open squares). For determination of p220 and eIF-4A, 150 μg of protein for each sample was analyzed by Western blotting as for Fig. 7. The eIF-4A (double triangles) and major p220 (closed triangles) bands were quantitated by densitometry with reflective illumination. All values are expressed relative to those for AS cells not treated with TCDD.

strated (35). The importance of the hypusine modification at Lys-50 of eIF-4D was similarly demonstrated in COS-1 cells (57). Overexpressing eIF-4B in COS-1 cells by 50-fold resulted in a general inhibition of translation (45). This approach has also been used for eIF-4E. Overexpression by as little as three- to eightfold led to a rapid growth phenotype reminiscent of oncogenically transformed cells (12, 40). The absence of this phenotype in cells transformed with the same vectors expressing the [Ala^{53}]eIF-4E variant provides in vivo evidence that phosphorylation at Ser-53 is essential for eIF-4E activity.

The second approach, to reduce the level of an initiation factor with AS RNA technology, has not been used prior to the study reported here. AS RNA is thought to inhibit expression of an mRNA by the formation of an RNA duplex which either inhibits its processing and transport from the nucleus, prevents its translation, or enhances its degradation (24). Melton (44) used AS RNA in *Xenopus* oocytes to inhibit β-globin translation and found that sequences complementary to the 5′ noncoding region or the 5′ region including the translation initiation site were more inhibitory than those complementary to the 3′ coding or 3′ noncoding regions. Izant and Weintraub (31) observed maximal inhibition of thymidine kinase expression in LTK$^-$ cells when they used AS RNA directed against the 5′ untranslated portion of the mRNA. On the basis of these results, we constructed the vector to express RNA which was complementary to 20 nt at the 5′ terminus of eIF-4E mRNA.

Whether because of the level of AS RNA in the cell or because of the site of complementarity, the expression of AS RNA against eIF-4E mRNA produced marked effects in our system. The rate of cell division was slowed or stopped, depending on the level of expression (Fig. 1), the rate of protein synthesis was dramatically decreased in vivo and in vitro (Fig. 2 and 7), polysomes disaggregated (Fig. 3), and the levels of both eIF-4E mRNA (Fig. 4 and 5) and eIF-4E protein (Fig. 6) were decreased. The two methods of eIF-4E mRNA estimation gave qualitatively similar results and indicated that AS cells grown in 0.2 mg of G418 per ml contained 1.7- to 3-fold less mRNA than did control cells. Addition of the inducer further reduced the mRNA level 2- to 3-fold, giving an overall reduction of 3- to 10-fold compared with control cells. Contente et al. (10) achieved a comparable reduction of *rrg* mRNA, but the reduction varied greatly among clonal transformants. Griep and Westphal (25) used the simian virus 40 early promoter to express AS RNA complementary to portions of the mouse c-*myc* gene. Cells transformed with these constructs displayed a dramatic reduction in *myc* protein. Surprisingly, the total cellular levels of *myc* mRNA were not affected, suggesting that the effect was at the level of mRNA transport or translation as opposed to synthesis, splicing, or stability. In the present study, the loss of eIF-4E protein appeared to be more dramatic than the loss of eIF-4E mRNA (compare Fig. 6 with Fig. 4 and 5), suggesting that there may be translational effects in addition to the decrease in eIF-4E mRNA levels.

Another important feature of the system described here is the ability to regulate the level of AS RNA expression. Inducible promoters have been used in previous studies to express AS RNA with varying success. Holt et al. (30) made constructs expressing c-*fos* AS RNA under the control of the MMTV promoter. Addition of dexamethasone to stable transformants caused rapid production of AS RNA and inhibition of cell proliferation. On the other hand, AS RNA against the mRNA encoding the tissue inhibitor of metalloproteinases, directed from a metallothionine promoter, was found to be constitutive and not inducible by heavy metals (36). A heat shock promoter was used to generate AS RNA against *Drosophila hsp-26* mRNA, but only cells with high copy numbers of the vector exhibited reduced levels of *hsp-26* protein (43). In the present study, AS RNA levels were altered either by varying the G418 selection or by inducing the promoter with TCDD. These two methods provide a wide range of experimental flexibility. Cells may be maintained indefinitely with different dosages of the antisense-encoding DNA by culturing in different G418 concentrations. Because of the low level of constitutive expression of the promoter-enhancer combination, these cells exhibit characteristic growth rates, but rapid changes in AS RNA levels are not possible because establishment of new G418 concentrations must be conducted in stages over periods of days to weeks. Activation of the promoter by TCDD, by contrast, can be demonstrated in as little as 1 h (18). The combination of G418 and TCDD is required for maximal expression of AS RNA; without G418, the vector copy number decreases so that addition of TCDD has little effect (Fig. 8, lane 2 versus lane 3). On the other hand, if AS RNA expression had been constitutively high, we would not have been able to obtain transformants because of the lethal phenotype (Fig. 1A, closed squares).

The coordinate loss of eIF-4E and p220 in AS cells was unexpected and may open the way for studies to clarify the relationship between these two polypeptides. Originally, eIF-4E was independently detected as a polypeptide which would either cross-link to mRNA caps (59), reverse the inhibition of protein synthesis caused by poliovirus infection (62), or reverse the inhibition of cell-free translation caused

by cap analogs (28). Subsequently, Tahara et al. (60) found that high-molecular-weight complexes containing eIF-4E were effective in reversing poliovirus inhibition but that eIF-4E alone was not. p220 was first identified as a protein which was proteolytically cleaved coincident with the shutoff of protein synthesis caused by poliovirus infection (23). It was then found by Grifo et al. (26) and Edery et al. (17) to be a component of the high-molecular-weight complexes discovered by Tahara et al. (60). It is worth noting, however, that all such complexes have been isolated from cells or ribosomal extracts after treatment with 0.5 M KCl, and that attempts to isolate p220:eIF-4E complexes from whole cell lysates without high-salt treatment have failed (9). Thus, the association between these two proteins may be transient and occur only during a specific stage of initiation. The phosphorylation of eIF-4E may also have a bearing on the association of p220 and eIF-4E. The nonphosphorylatable [Ala^{53}]eIF-4E variant does not become bound to the 48S initiation complex (33). Assuming that eIF-4E is bound to the 48S complex via p220, this may mean that phosphorylated eIF-4E has a higher affinity for p220 than does unphosphorylated eIF-4E. Consistent with this idea, Tuazon et al. (63) found that phosphorylated eIF-4E could be isolated in a complex with p220, but that a similar complex was not formed when unphosphorylated eIF-4E was added. This would be reminiscent of eIF-2B having a higher affinity for eIF-2α-P than for eIF-2α (54). These results, coupled with the knowledge that the phosphate group of eIF-4E turns over more rapidly than does the polypeptide chain (53), suggest a model in which the association of eIF-4E with p220 is in dynamic equilibrium and is determined by eIF-4E phosphorylation.

As noted above, eIF-4F is isolated as a complex of three components: eIF-4A, eIF-4E, and p220. The fact that eIF-4A does not decrease under the same conditions which lead to a complete loss of eIF-4E and p220 (Fig. 9) suggests that the eIF-4E and p220 components may be more closely associated, physically, functionally, or from a regulatory standpoint. In fact, eIF-4A is present at a considerably higher level than is either eIF-4E or p220 (16). Also, some purification schemes yield a p220:eIF-4E complex with no eIF-4A (9, 22, 39).

Although there are several possibilities which might explain why a reduction of p220 is correlated with a loss in eIF-4E, the most likely explanation is that p220 is degraded by cellular proteases unless it is complexed with eIF-4E. This interpretation, as opposed to the hypothesis that eIF-4E affects p220 expression at the transcriptional or translational level, is supported by the nearly simultaneous loss of eIF-4E and p220 (Fig. 8). Such a mechanism could potentially reconcile the observations of Trachsel et al. (62), who found that the addition of purified eIF-4E was sufficient to stimulate translation in extracts of poliovirus-infected cells, with those of Grifo et al. (26), who found that eIF-4F rather than eIF-4E was required. Perhaps poliovirus infection initially causes an inactivation or sequestration of eIF-4E, and the failure of eIF-4E and p220 to interact leads to the degradation of p220. Thus, addition of eIF-4E alone could restore translation to cell extracts at early times during the course of viral infection but not later, when the p220 component is degraded.

From a practical standpoint, the AS cells provide a model system for the study of translation in mammalian cells and cell extracts which are deficient in eIF-4E and p220, akin to the yeast cell-free system containing a temperature-sensitive eIF-4E variant (2). It is yet to be determined whether other translational components are reduced in the AS cells, but the ability of highly purified eIF-4F to restore translation (Fig. 7A) together with the unchanged levels of eIF-4A (Fig. 9) argues against this. Previously, poliovirus-infected cells and their lysates, or cell-free translation systems derived from components of poliovirus-infected and normal cells, have been useful in investigating the role of p220 and eIF-4F (21, 46). An advantage of the AS cells and their extracts for this purpose is that the numerous biochemical and morphological changes occurring in poliovirus-infected cells (37) would not be complicating factors. The system will be especially suitable for studies of mRNA competition and discrimination both in vitro and in vivo.

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Inhibition of Human Immunodeficiency Virus Type 1 Multiplication by Antisense and Sense RNA Expression

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Human immunodeficiency virus type 1 (HIV-1) primarily infects CD4⁺ lymphocytes and macrophages and causes AIDS in humans. Retroviral vectors allowing neomycin phosphotransferase (*npt*) gene expression were engineered to express 5′ sequences of HIV-1 RNA in the antisense or sense orientation and used to transform the human CD4⁺ lymphocyte-derived MT_4 cell line. Cells expressing antisense or sense RNA to the HIV-1 *tar* mRNA leader sequence, as part of the 3′ untranslated region of the *npt* mRNA, remained sensitive to HIV-1 infection. In contrast, resistance to HIV-1 infection was observed in cells expressing antisense RNA to the HIV-1 primer-binding site or to the region 5′ to the primer-binding site as part of the 3′ region of the *npt* mRNA. Cells expressing the *tar* mRNA leader sequence in the sense orientation as a precise replacement of the 5′ untranslated region of *npt* mRNA were also resistant to HIV-1. These results indicate that sense and antisense approaches can be used to interfere with HIV-1 multiplication.

AIDS is caused by a retrovirus, human immunodeficiency virus type 1 (HIV-1) (3). This virus primarily infects CD4⁺ lymphocytes and macrophages. Although other cell types also become infected, HIV-1 seems to preferentially kill CD4⁺ lymphocytes (17). These lymphocytes, as well as the other fully differentiated cells within the hematopoietic system, including mature myeloid and lymphoid cells, are maintained by the continuous proliferation and differentiation of relatively small numbers of stem cells. Thus, if a stem cell could be made resistant to HIV-1 by the transfer of anti-HIV-1 constructs with retroviral vectors, then its progeny, including cells within the immune system, might also be HIV-1 resistant. Toward this aim, retroviral vectors are being developed and tested in vitro and in vivo with hematopoietic stem cells, fibroblasts, hepatocytes, and endothelial cells (6, 10, 16, 29).

It may be possible to interfere with HIV-1 multiplication at the level of replication and/or gene expression by using antisense (35) or sense (26, 33) RNA strategies. Antisense RNA, complementary to a specific portion of an RNA molecule, could, upon hybridization with target RNA sequences, disrupt reverse transcription, processing, translation, and/or transport of this RNA. Antisense RNAs have been shown to alter the expression of selected genes in several cell systems, including bacteria (25), *Xenopus* oocytes (22), *Drosophila* embryos (30), and plant (8) and mammalian (12) cells. Avian retrovirus (34) and human T-cell lymphotropic virus type I (36) replication has also been shown to be inhibited in antisense RNA-expressing cell lines. The degree of inhibition obtained in these studies was variable and depended on many factors, including size, hybridization location, secondary structure, and level of expression of both the antisense RNA and the target mRNA whose expression was being modulated.

A sense RNA approach has been used to block replication of the genome of a plant RNA virus by using the origin of replication located at the 3′ end of the genome as a competitive inhibitor for viral replicase (26). RNA-RNA and RNA-protein interactions are crucial for HIV-1 replication, *trans* activation, transcription, transport, translation, and packaging, and the HIV-1 RNA sequences involved in these interactions are known (3). RNA containing these sequences in a sense orientation followed by non-HIV-1 sequences could compete with HIV-1 mRNAs for binding of RNA and/or protein and result in inhibition of HIV-1 multiplication.

HIV-1 replication takes place by using virally encoded reverse transcriptase and cellular $tRNA_3^{Lys}$, which binds to the HIV-1 primer-binding site (PBS) via an 18-nucleotide-long sequence complementarity (3). This region is highly conserved among all HIV-1 isolates. Upon cDNA synthesis to the 5′ region of the HIV-1 RNA and RNase H degradation of the template RNA, this cDNA hybridizes to the complementary sequences present at the 3′ end of the HIV-1 RNA. Interference at the level of initiation of HIV-1 replication could be achieved by expression of antisense RNA to the PBS that could compete with the primer $tRNA_3^{Lys}$ for binding to the HIV-1 PBS. Antisense RNA to the region 5′ to the HIV-1 PBS could further prevent elongation of HIV-1 replication. In fact, exogenously added short synthetic oligodeoxyribonucleotides have been shown to inhibit HIV-1 multiplication (21). Alternatively, interference with HIV-1 replication may be achieved by expressing sense RNA to the 5′ sequences of HIV-1 that could compete with HIV-1 RNA during the strand-switching process.

HIV-1 mRNAs used for synthesis of various HIV-1 proteins contain 287 identical nucleotides at their 5′ end. This region contains a *trans* activation-responsive (TAR) element that allows for a 1,000-fold increase in HIV-1 gene expression upon interaction with the HIV-1 *tat* protein. Interference with HIV-1 gene expression at the level of translation may be achieved by expressing antisense RNA to the 5′ leader sequence of HIV-1. Similarly, sense RNA to HIV-1 leader sequence could inhibit gene expression (i.e., *trans* activation) by acting as a competitor for factors which normally bind to the HIV-1 mRNAs.

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In this study, various retroviral vectors were engineered to express chimeric RNA containing antisense or sense RNA to the 5′ sequence of HIV-1 RNA in human CD4⁺ lymphocyte-derived cell lines, and cells were tested for susceptibility to HIV-1 infection.

MATERIALS AND METHODS

Enzymes and chemicals. All restriction enzymes were purchased from Bethesda Research Laboratories, Inc. T4 DNA ligase, T4 polynucleotide kinase, and DNA polymerase I Klenow fragment were obtained from Pharmacia. Calf intestinal alkaline phosphatase and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were obtained from Boehringer Mannheim. Isopropyl-thio-β-D-galactoside was obtained from P-L Biochemicals, Inc. Bovine calf serum, geneticin (G418), antibiotics (containing penicillin, streptomycin, and amphotericin B [Fungizone]), L-glutamine, α-minimal essential medium and RPMI 1640 medium were purchased from GIBCO. P24 antigen detection kit was obtained from Abbott Laboratories.

Plasmid constructions. Unless stated otherwise, all recombinant DNA techniques were performed as described in reference 19. Site-specific in vitro mutagenesis was done essentially as described in reference 15 with minor modifications (13). DNA sequences were confirmed by previously described procedures (23, 31). Nucleotide sequences from the HIV-1_{HXB2} strain (28) were used to design all antisense and sense RNA-expressing vectors.

Moloney murine leukemia virus-derived retroviral vectors pUCMoTN and pBRMoTN (18) were used in this study. In these vectors, the 3′ untranslated region of neomycin phosphotransferase (*npt*) mRNA contains about 1,400 nonessential nucleotides between the *Asu*II and *Cla*I sites. These nucleotides were therefore deleted in the vector pUCMoTNΔAC by *Asu*II-to-*Cla*I deletion and religation at these compatible sites.

pUCMoTN-PBS(−) and pUCMoTN-5′PBS(−) vectors were constructed as outlined below. The following oligonucleotides, PBS(−) and 5′PBS(−), containing the reverse complement of the sequences desired to be present on the RNA were synthesized:

PBS(−): pCTCTATAGGCTTCAGCTGGGGGtcgaaTT
TTTTTTTGGCGCCCGAACAGGGACTTGAATTTTT
atcgaatCCCCCAACTAGAGCCTGGACCA

5′PBS(−): pCTCTATAGGCTTCAGCTGGGGGatcgaatG
GGGGTTGTGTGGAAAATCTCTAGCAGGGGGGGtcgaa
aCCCCCAACTAGAGCCTGGACCA

Nucleotides shown with a double underline can base pair with each other, nucleotides shown in italics with an underline cannot base pair with each other, nucleotides shown in boldface correspond to the HIV-1 PBS or to the region 5′ to the PBS, and nucleotides shown in lowercase letters correspond to restriction sites (*Asu*II or *Cla*I) inserted for future cloning purposes.

These oligonucleotides were used separately for in vitro mutagenesis of the vector M13 containing the *Sph*I-to-*Sst*I fragment of the vector pUCMoTNΔAC. The correct clones were identified by restriction enzyme analysis and confirmed by DNA sequencing. The *Sph*I-to-*Sst*I fragment, containing the DNA sequences mutagenized with the oligonucleotide PBS(−) or 5′PBS(−), was then used to replace the unmutagenized portion of the pUCMoTN vector. The resulting vectors were called pUCMoTN-PBS(−) and pUCMoTN-5′PBS(−).

pUCMoTN-L(5′+) vector was constructed as follows. In this vector, the 5′ leader sequence of the *npt* mRNA was precisely replaced with the HIV-1 *tat* mRNA leader sequence (12a). Essentially, five double-stranded oligonucleotides were synthesized for this purpose. Starting from the 5′ end, these oligonucleotides contained *Pst*I and *Cla*I restriction sites, part of the herpes simplex virus thymidine kinase (*tk*) promoter region from the *Mlu*I site to nucleotide −1, nucleotides +1 to the AUG of the HIV-1 *tat* mRNA 5′ leader sequence, part of the *npt* coding region from AUG to *Eag*I, and *Asu*II and *Eco*RI restriction sites. These oligonucleotides were ligated together and cloned in M13 at the *Pst*I and *Eco*RI sites. Correct clones were identified by restriction enzyme analysis, and the nucleotide sequence of the resulting vector, M13-L, was further confirmed by DNA sequencing. The *Mlu*I-to-*Eag*I fragment of M13-L was then used to replace the unmutagenized portions in a plasmid containing the *Eco*RI-*Sph*I fragment of pUCMoTN. The *Eco*RI-*Sph*I fragment of the resulting vector, containing the HIV-1 *tat* mRNA leader sequence, was then cloned in the pUCMoTN vector at the *Eco*RI and *Sph*I sites. The resulting vector was called pUCMoTN-L(5′+).

pBRMoTN-L(3′+) vector was constructed by cloning the *Asu*II-to-*Cla*I fragment of the M13-L vector by blunt-end ligation at the *Bam*HI site in the vector pBRMoTN. The correct clones containing the HIV-1 *tat* mRNA leader sequence in the sense orientation were selected by restriction enzyme analysis.

The vector pUCMoTN-L(−), containing HIV-1 *tat* mRNA 5′ leader sequence in the antisense orientation, was constructed by cloning the *Asu*II-to-*Cla*I fragment of M13-L into the pUCMoTN vector at the *Asu*II-to-*Cla*I sites. The correct clones containing these restriction sites were selected by restriction enzyme analysis.

Cell lines. Psi-2 (20) and PA317 (24) packaging cell lines were cultured in α-minimal essential medium supplemented with 2 mM L-glutamine, antibiotics (penicillin, 100 units/ml; streptomycin, 100 μg/ml; amphotericin B, 0.25 μg/ml), and 10% fetal calf serum at 37°C in a humidified atmosphere with 6% CO_2. The human CD4⁺ lymphocyte-derived MT_4 (suspension) cell line was cultured in RPMI 1640 medium supplemented with L-glutamine (2 mM), antibiotics, and fetal calf serum (10%) at 37°C in an atmosphere of 6% CO_2.

Transfection and infection of mammalian cell lines. Six hours after 2×10^5 Psi-2 cells were seeded on 60-mm tissue culture dishes in 4 ml of medium, the cultures were transfected with 1 μg of plasmid DNA by previously described procedures (11, 37). Sixteen hours later, the cells were washed and cultured. Once confluent, the cells were trypsinized and transferred to 100-mm tissue culture dishes and cultured in medium containing antibiotics, 10% calf serum, and G418 (200 μg/ml). The selective medium was changed every 4 to 5 days, and the number of colonies was counted after 14 days.

Vector particles released from transformed Psi-2 cells at 50 to 100% confluency were obtained by filtering the culture medium through a 0.22-μm-pore-size filter. These particles were used to infect PA317 cells as described previously (14). Essentially, 2×10^5 cells in 4 ml of medium were seeded for 6 h in 60-mm tissue culture dishes, after which this medium was replaced by 1 ml of medium containing 8 μg of Polybrene per ml and 100 μl of vector particles. After 2 h of incubation at 37°C, 3 ml of medium was added and the incubation was continued for 16 more hours. Cells were then trypsinized and transferred to 100-mm tissue culture dishes in the presence of selective medium containing 200 μg of

G418 per ml. The selective medium was changed every 4 to 5 days, and the number of colonies was counted after 14 days.

The transformed PA317 cell lines releasing retroviral vector particles were used to infect MT_4 cells by cocultivation (1). MT_4 (10^6) cells in 10 ml of RPMI 1640 medium containing L-glutamine, antibiotics, and 10% fetal calf serum were placed on a 100-mm tissue culture dish containing transformed PA317 cells (50% confluent). After 24 h of cocultivation at 37°C, the infected MT_4 cells in suspension were transferred to a 100-mm petri dish and incubated in the presence of the above medium supplemented with 400 μg of G418 per ml. This selective medium was changed every 4 to 5 days for up to 3 to 4 weeks (time required for untransformed cells to die). The pool of stably transformed MT_4 cells (without further cloning) was then used for RNA analysis and subjected to HIV-1 challenge.

Northern (RNA) blot and slot-blot analysis. RNA was isolated as described in reference 4. Essentially, cells were lysed in a denaturing solution containing guanidinium isothiocyanate, and the total RNA was pelleted by centrifugation through a CsCl cushion. For Northern blot analysis, total cellular RNA (9 μg) was loaded onto a 1.5% formaldehyde gel and subjected to electrophoretic separation. Fractionated RNA was capillary blotted to a GeneScreen Plus membrane (Dupont), which was then probed with the ^{32}P-labeled *Sstl-Sstl* fragment (188 nucleotides long) of pUC MoTN-L(5′+) vector.

For slot-blot analysis, RNA was isolated as described in reference 5. Essentially, total RNA was recovered from cell lysates by a single extraction with a mixture of phenol and chloroform. Total cellular RNA (9 μg) was immobilized onto a GeneScreen Plus membrane by gentle suction with a blotting manifold (Bethesda Research Laboratories). The membrane was then probed with the ^{32}P-labeled 188-nucleotide-long *Sstl-Sstl* fragment.

HIV-1 infection and p24 antigen detection. The titer of the HIV-1_{IIIB} strain replicated in MT_4 cells (32) was determined by using the same cell line. Without further propagation in our laboratory, the titer of the HIV-1_{NL4-3} strain, replicated in A3-01 cells transfected with the NL4-3 provirus DNA, was also determined by using MT_4 cells. For both HIV-1 strains, 10 μl of virus preparation was used to infect 10^6 MT_4 transformants (G418 resistant) in 1 ml of RPMI 1640 medium containing L-glutamine, antibiotics, 10% fetal calf serum, and 400 μg of G418 per ml. The cells were incubated at 37°C in an atmosphere of 6% CO_2. Two hours later, the cells were washed to remove unadsorbed virus particles and the incubation was continued. Culture supernatant (100 μl) was collected at various intervals and tested for p24 antigen (24-kDa HIV-1 *gag* gene product) production by enzyme-linked immunosorbent assay (ELISA). An optical density value of 1 corresponds to 200 pgs of HIV-1 p24 antigen. The maximum optical density value reported is 2 since values greater than this could not be accurately measured owing to the sensitivity of the ELISA reader.

RESULTS AND DISCUSSION

Moloney murine leukemia virus-derived retroviral vectors pBRMoTN, pUCMoTN (18), and pUCMoTNΔAC, containing the herpes simplex virus *tk* promoter driving *npt* gene expression (conferring G418 resistance), were used in this study. These vectors were engineered to express antisense or sense RNA molecules to HIV-1 as part of the 3′ or 5′ untranslated region of the *npt* mRNA. These vectors were used to generate transformed CD4$^+$ lymphocyte-derived MT_4 cell lines, which were then tested for antisense or sense RNA expression and for their susceptibility to HIV-1 infection.

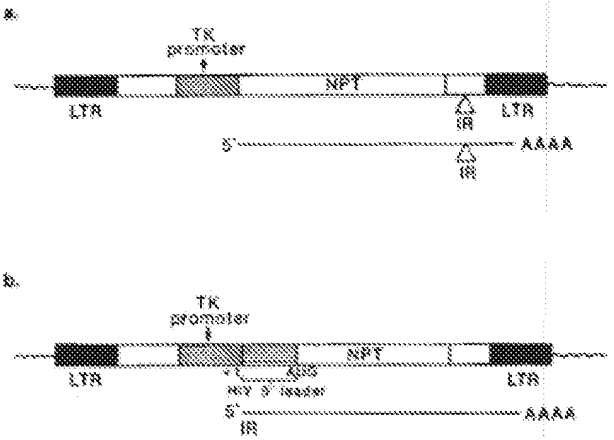

FIG. 1. Retroviral vectors expressing antisense or sense RNA molecules to HIV-1. (a) Interfering RNA (IR) corresponds to antisense RNA to the HIV-1 PBS [MoTN-PBS(−)], to antisense RNA to the region 5′ to the PBS [MoTN-5′PBS(−)], or to the HIV-1 *tat* mRNA 5′ leader sequence cloned in the antisense [MoTN-L(−)] or sense [MoTN-L(3′+)] orientation. These sequences were cloned as part of the 3′ untranslated region of the *npt* mRNA. (b) Interfering RNA corresponds to the 5′ leader sequence of HIV-1 *tat* mRNA that was used to precisely replace the 5′ leader sequence of *npt* mRNA [MoTN-L(5′+)]. TK, thymidine kinase; LTR, long terminal repeat.

Antisense RNA-expressing vectors. Antisense RNAs to HIV-1 were expressed as part of the 3′ untranslated region of *npt* mRNA between the *npt* stop codon and the polypurine tract (PPT). The following retroviral vectors were constructed (Fig. 1a): pUCMoTN-PBS(−), expressing antisense RNA to the HIV-1 PBS (18 nucleotides long); pUCMoTN-5′PBS(−), expressing antisense RNA to the 18-nucleotide-long region 5′ to the HIV-1 PBS; and pUCMoTN-L(−), expressing antisense RNA to the HIV-1 *tat* mRNA 5′ leader sequence. This leader sequence contains 343 nucleotides, of which the first 287 nucleotides are common to all HIV-1 mRNAs. Antisense RNAs to the HIV-1 PBS (18 nucleotides long) and to the 18-nucleotide-long region 5′ to the PBS are of relatively short length. Therefore, to render them available for hybridization to the HIV-1 target mRNA, it seemed important to express them as part of a loop structure. This was achieved by selecting within the 3′ untranslated region of *npt* mRNA a stable stem-and-loop structure as predicted by Zucker and Streigler's computer program (38) and replacing the sequences within this loop with the HIV-1 antisense RNA sequences. To further ensure that a stem structure will form and that the antisense RNA sequences to HIV-1 will be located at the center of the loop, base pairing in the stem region was increased by the insertion of 9 bp and the base of the loop was changed by the insertion at either end of 5 nucleotides that cannot base pair with each other.

Sense RNA-expressing vectors. The following retroviral vectors were constructed: pUCMoTN-L(5′+), expressing HIV-1 *tat* mRNA leader sequence (+1 to AUG; 343 nucleotides) precisely replacing the *npt* mRNA 5′ leader sequence (Fig. 1b); and pBRMoTN-L(3′+), expressing HIV-1 *tat* mRNA leader sequence as part of the 3′ untranslated region of *npt* mRNA (Fig. 1a).

Transformation of MT_4 cell lines with retroviral vector

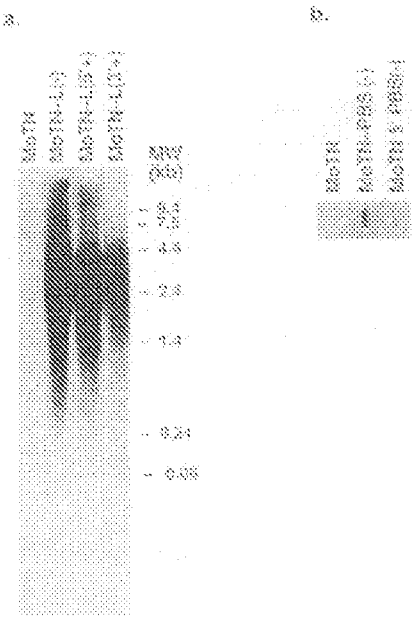

FIG. 2. Antisense and sense RNA levels in MT_4 transformants. (a) Northern blot analysis of antisense and sense RNA to the HIV-1 *tat* mRNA 5′ leader sequence expressed in MT_4 cells containing MoTN-L(−), MoTN-L(5′+), and MoTN-L(3′+). MT_4 cells containing MoTN served as a control. MW, molecular weight. (b) Slot-blot analysis of antisense RNA to the HIV-1 PBS or to the region 5′ to the PBS expressed in MT_4 cells containing MoTN-PBS(−) or MoTN-5′PBS(−), respectively.

particles. The retroviral vectors engineered to express antisense [pUCMoTN-PBS(−), pUCMoTN-5′PBS(−), and pUCMoTN-L(−)] and sense [pUCMoTN-L(5′+) and pBRMoTN-L(3′+)] RNAs to HIV-1, as well as the vector (pUCMoTN) lacking the test sequences, were used to transfect the ecotropic Psi-2 packaging cell line. The retroviral vector particles [MoTN-PBS(−), MoTN-5′PBS(−), MoTN-L(−), MoTN-L(5′+), MoTN-L(3′+), and MoTN] released from these transformants were used to infect the amphotropic PA317 packaging cell line. These transformants, releasing amphotropic retroviral vector particles, were then used in cocultivation experiments to infect the MT_4 cell line, and stable MT_4 transformants were selected by growth for up to 1 month in medium containing G418.

Antisense and sense RNA levels in MT_4 transformants. Northern blot analysis of RNA isolated from the MT_4 transformants was performed to assess the level of expression of various antisense and sense RNA molecules to HIV-1 (Fig. 2a). The probe used in these experiments was the ^{32}P-labeled 188-nucleotide-long *Sstl-Sstl* fragment of pUC MoTN-L(5′+) containing part of the HIV-1 *tat* mRNA 5′ leader sequence, including the HIV-1 PBS and the 18-nucleotide-long region 5′ to the PBS. This probe should hybridize to the vector and *npt* mRNAs containing the HIV-1 antisense or sense RNA sequences and not to RNAs lacking these sequences. As shown in Fig. 2a, the presence of RNAs containing antisense or sense RNA molecules to HIV-1 was clearly detectable in the MT_4 transformants containing MoTN-L(−), MoTN-L(5′+), and MoTN-L(3′+). It is noteworthy that similar levels of Moloney murine leukemia virus long terminal repeat and *tk* promoter-driven RNAs containing antisense and sense RNA sequences to HIV-1 were expressed from all three vectors (Fig. 2a; data not shown). Antisense RNA to the HIV-1 PBS or to the region 5′ to the PBS could not be detected in this experiment (data not shown), most likely because base pairing between these antisense RNAs and the probe was only over 18 nucleotides; for other sense and antisense RNAs, whose expression could be detected, base pairing occurred over a stretch of 188 nucleotides. Slot-blot analysis, at a lower stringency, was therefore performed with the same probe to detect the presence of RNAs containing the 18-nucleotide-long antisense RNA sequences to the HIV-1 PBS and to the region 5′ to the PBS. As shown in Fig. 2b, the presence of HIV-1 antisense RNAs expressed in the MT_4 transformants containing MoTN-PBS(−) and MoTN-5′PBS(−) was clearly detectable under these conditions.

It is noteworthy that cells transformed with the MoTN-L(5′+) vector allowing *npt* gene expression under control of the *tk*-TAR fusion promoter were G418 resistant and allowed high-level expression of *npt* mRNA (Fig. 2a). These results indicate that this fusion promoter can allow constitutive gene expression in the absence of *tat* protein.

Challenge of antisense or sense RNA-expressing MT_4 cells with HIV-1. Retroviral vectors engineered in this study were designed to express HIV-1 antisense or sense RNA sequences corresponding to HIV-1_{HXB2}, which is one of the clones obtained from HIV-1_{IIIB}, a pooled virus. Nucleotide sequences of other viruses present in HIV-1_{IIIB} are unknown. These isolates, depending on their nucleotide sequence, might escape inhibition by the antisense or sense RNAs expressed from retroviral vectors engineered in this study and multiply. This, in turn, could obscure the protective effect of antisense or sense RNA molecules to the HIV-1_{HXB2} isolate. Therefore, the ideal solution would be to use homogeneous virus released from a cell line containing a cloned provirus whose sequence is identical or at least very similar to that of HIV-1_{HXB2}. Subsequently, one such cloned virus, HIV-1_{NL4-3}, was used in our studies. Its sequence within nucleotides +1 to +287 is quite similar to that of HIV-1_{HXB2}. This sequence identity is 100% for the PBS and for the 18-nucleotide-long region 5′ to the PBS. Both HIV-1_{IIIB} and HIV-1_{NL4-3} were used in HIV-1 challenge experiments described below.

HIV-1 resistance of antisense RNA-expressing MT_4 transformants. MT_4 cells transformed with MoTN-PBS(−), MoTN-5′PBS(−), and MoTN-L(−) vectors expressing antisense RNAs to HIV-1 were challenged with HIV-1_{IIIB} (Fig. 3a and b) and HIV-1_{NL4-3} (Fig. 3c). Infection of MT_4 cells transformed with MoTN vector lacking the test sequences served as a control. After infection, HIV-1 production was monitored at various time intervals by measuring the level of p24 antigen (HIV-1 *gag* gene product) in cell culture supernatant.

The MT_4 transformants expressing antisense RNA to the PBS or to the region 5′ to the PBS were partially resistant to both strains of HIV-1 tested, HIV-1_{IIIB} (Fig. 3a) and HIV-1_{NL4-3} (Fig. 3c), as virus production was decreased significantly. The expression of these antisense RNAs at the center of a stable loop structure might have contributed to the observed partial resistance. Using retroviral vectors allowing strong constitutive and/or *tat*-inducible expression, it is tempting to speculate that the vector MoTN-PBS(−) will be able to confer resistance to all HIV-1 isolates as this region is highly conserved.

The cells expressing antisense RNA to the HIV-1 *tat* mRNA leader sequence (343 nucleotides long) failed to prevent multiplication of both strains of HIV-1 tested (Fig. 3b and c). This negative result could be explained in several

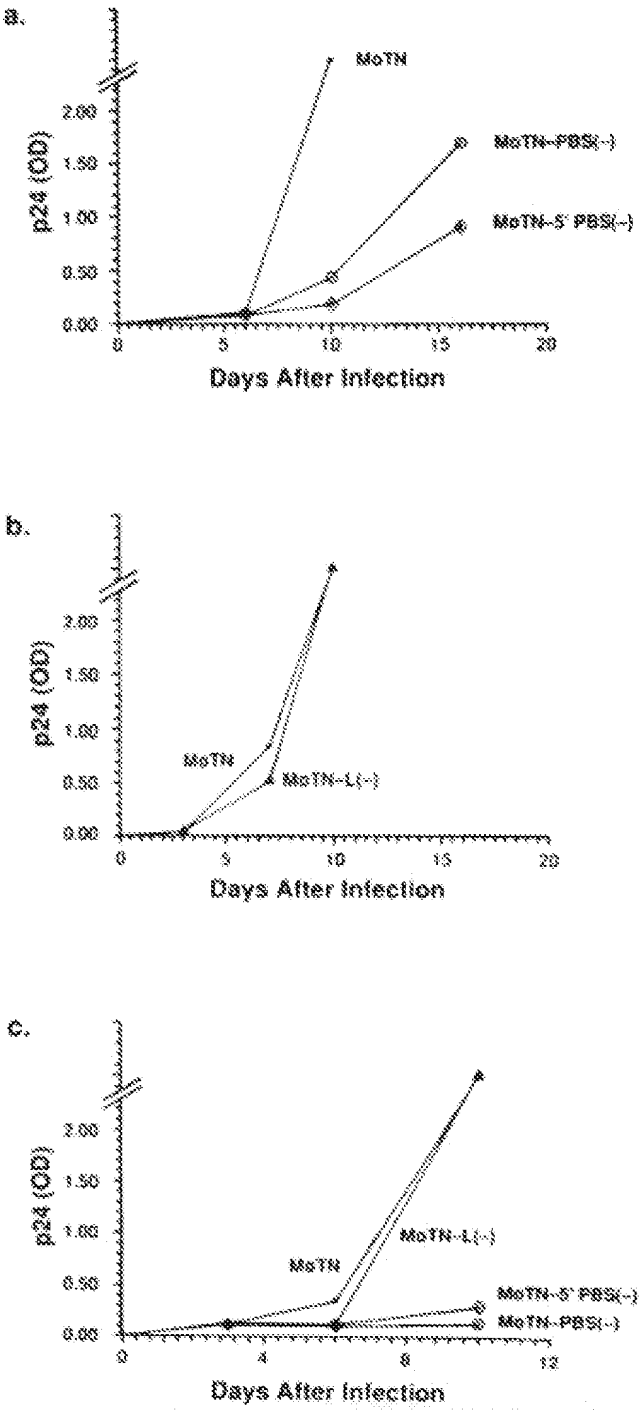

FIG. 3. Susceptibility of MT_4 cells expressing antisense RNAs to infection by both HIV-1_{IIIB} and HIV-1_{NL4-3}. MT_4 cells transformed with MoTN (▽), MoTN-PBS(−) (○), MoTN-5′PBS(−) (◇), or MoTN-L(−) (△) were infected with HIV-1_{IIIB} (a and b) or with HIV-1_{NL4-3} (c), and the level of p24 antigen released in the culture supernatant was determined by ELISA at various times after infection. Values greater than 2 are shown at an arbitrarily chosen point. OD, optical density.

ways. (i) The synthetic oligonucleotides containing the HIV-1 *tat* mRNA leader sequence include part of the *tk* promoter region (*Mlu*I to nucleotide −1; 13 nucleotides long) and part of the *npt* mRNA (AUG to *Eag*I; 37 nucleotides long). These sequences, when present in an antisense orientation at the 3′ end of the *npt* mRNA, could base pair with the complementary sequences located at the 5′ end of this *npt* mRNA. Such intramolecular base pairing might prevent interaction between antisense RNAs and target HIV-1 mRNAs. (ii) The antisense RNA approach might be impeded by nucleotide sequence variations between the antisense RNA and the pooled virus (HIV-1_{IIIB}) and, to a lesser extent, the cloned virus (HIV-1_{NL4-3}) used. (iii) The TAR sequence present at the 5′ end of all HIV-1 mRNAs forms a stable stem-and-loop structure (27) and binds to the *tat* protein (7). This region, therefore, might not be available for hybridization to the antisense RNA. (iv) Finally, TAR-containing mRNAs might be compartmentalized and/or translated in the presence of *tat* protein via a unique mechanism (2, 9) that is not subject to inhibition by an antisense RNA approach.

HIV-1 resistance of sense RNA-expressing MT_4 transformants. MT_4 cells transformed with MoTN-L(5′+) or MoTN-L(3′+) expressing sense RNA to the HIV-1 *tat* mRNA 5′ leader sequence as part of the 5′ or 3′ region of *npt* mRNA, respectively, were subjected to challenge by HIV-1_{IIIB} (Fig. 4a) and HIV-1_{NL4-3} (Fig. 4b). Cells containing the MoTN vector lacking test sequences served as controls. MT_4 cells transformed with MoTN-L(5′+), expressing the HIV-1 *tat* mRNA leader sequence as a precise replacement of the *npt* mRNA leader sequence, were quite resistant to HIV-1_{IIIB} and HIV-1_{NL4-3} infection. However, expression of sense RNA to HIV-1 within the 3′ region of *npt* mRNA had no protective effect as HIV-1 production in these cells transformed with MoTN-L(3′+) was similar to that obtained from cells containing MoTN (Fig. 4).

Differences in resistance to HIV-1 infection conferred by retroviral vectors in which the HIV-1 sequences are located within the 5′ leader sequence or the 3′ untranslated region of the *npt* gene might be due to differences in the ability of these transcription units to be up-regulated by the HIV-1 *tat* protein. For *trans* activation by the *tat* protein at the level of transcription, a particular spacing is required between the TAR region and the transcription factor-binding sites present within the promoter region (32). This spacing is conserved in the vector MoTN-L(5′+) but is too large in the vector MoTN-L(3′+). Thus, *tat*-inducible *trans* activation is only expected to occur from the *tk*-TAR promoter present in the vector MoTN-L(5′+). Levels of sense RNA to HIV-1 expressed in the absence of *tat* protein in cells containing MoTN-L(5′+) or MoTN-L(3′+) were very similar (Fig. 2a). However, because of *trans* activation in the presence of *tat* protein, the level of HIV-1 sense RNA expressed from the vector MoTN-L(5′+) could be higher than that expressed by the vector MoTN-L(3′+). The stability of these two sense RNA molecules could be different as well.

Alternatively, binding of the HIV-1 *tat* protein to the TAR sequence (7) is expected to decrease the amount of *tat* protein available for *trans* activation of HIV-1 gene expression (33). If one assumes that the binding of *tat* protein to the TAR sequence located at an internal position in RNAs expressed from the vector MoTN-L(3′+) is not possible, inhibition of *trans* activation of HIV-1 gene expression would only occur with RNAs containing the TAR sequence at the 5′ end expressed from the vector MoTN-L(5′+). Moreover, only RNAs containing the *tat* mRNA leader

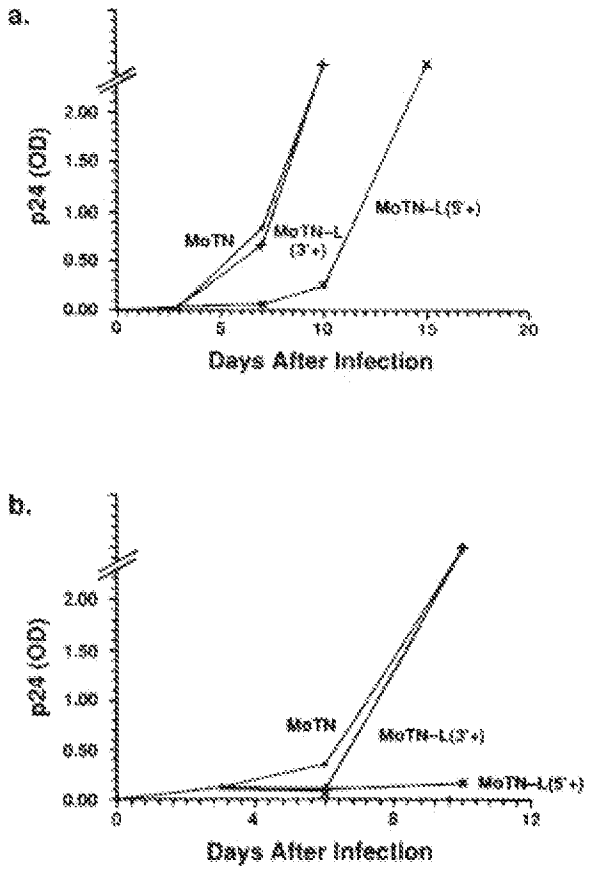

FIG. 4. Susceptibility of MT_4 cells expressing sense RNAs to infection by HIV-1_{IIIB} and HIV-1_{NL4-3}. MT_4 cells transformed with MoTN (•), MoTN-L(5'+) (×), or MoTN-L(3'+) (+) were infected with HIV-1_{IIIB} (a) or HIV-1_{NL4-3} (b), and the level of p24 antigen released in the culture supernatant was determined by ELISA at various times after infection. For details, see the legend to Fig. 3.

sequence as a precise replacement of the *npt* mRNA leader sequence, expressed from MoTN-L(5'+), are expected to compete with translation of HIV-1 mRNAs.

Finally, during replication, possible strand switching onto the HIV-1 5' sequences contained in the sense orientation at either the 5' or the 3' end of the *npt* mRNA should have resulted in abortive replication. However, since no inhibition of HIV-1 multiplication was observed in cells containing MoTN-L(3'+), the sense RNA present at the 5' end of *npt* mRNA might interfere with HIV-1 multiplication at another level.

Sense RNA to HIV-1 *tat* mRNA leader in the retroviral vector MoTN-L(5'+) probably acts by binding to *tat* protein or to some other cellular factors. If so, this vector will inhibit multiplication of all HIV-1 isolates in which binding to these viral or cellular factors is maintained.

In conclusion, inhibition of HIV-1 multiplication was observed in CD4$^+$ lymphocyte-derived MT_4 cell lines transformed with retroviral vectors expressing antisense RNA to the PBS or to the region 5' to the PBS as part of a loop structure within the 3' untranslated region of *npt* mRNA. Inhibition was also evident with a vector expressing HIV-1 *tat* mRNA 5' leader sequence in the sense orientation as a precise replacement of the *npt* mRNA 5' leader sequence. The next step would be to determine at which step these sense and antisense RNAs interfere with the HIV-1 life cycle (i.e., replication, *trans* activation, or translation). Moreover, antisense RNAs expressed under the control of the Moloney murine leukemia virus long terminal repeat and the herpes simplex virus *tk* promoter might not be able to keep pace with HIV-1 mRNAs produced as a result of *trans* activation by the *tat* protein. These results might therefore be improved by using retroviral vectors allowing strong constitutive and/or *tat*-inducible gene expression.

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Nucleolar protein P120 and its targeting for cancer chemotherapy.

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Identification of the Gl-P120 antigen with the aid of the monoclonal antibody to its "human-specific epitope" has resulted in rapid development of information on its molecular biology. With the monoclonal antibody, it rapidly became possible to identify and subsequently sequence its cDNA and with cDNA clones to isolate and sequence its genomic DNA. It was demonstrated that the protein had 4 major domains: a basic domain, an acidic domain, a hydrophobic and methionine-rich domain and a domain rich in cysteine and proline residues. In addition to a nuclear recognition signal, the epitope region is juxtaposed to phosphorylation sites. The epitope region contains the sequence Gln-Ala-Ala-Ala-Gly-Ile-Asn-Trp which is unique to the human P120 molecule; this may be a site for drug attack either by analogs to the region or by novel constructs based on antisense oligonucleotides. When tumor cells were transfected with antisense constructs of the P120 gene, growth rates were markedly reduced. 3T3 cells transformed by transfection with the P120 gene reverted to a nontransformed state by subsequent transfection and activation of a P120 antisense construct. Opportunities for control of malignant cells with antisense oligonucleotides are currently under study.

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Antisense RNA Therapy for CML — An Hypothesis

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Abstract — CML, a myeloproliferative clonal disorder of myeloid stem cells, is characterized by the consistent presence of a bcr — c-abl fusion gene which is formed as a result of a translocation of the c-abl gene from chromosome 9 to downstream of the bcr gene on chromosome 22 (ph'). Current approaches to the treatment of CML are chemotherapy (conventional or aggressive with immuno-modulators) and bone marrow transplantation (BMT). Neither of the above treatment modalities results in long-term remission or cure. Hence, an alternative approach which aims at correcting the genetic defect should be considered. Taking advantage of the consistent abnormal presence of the bcr — c-abl gene in the treated and untreated CML patients at all stages, a gene therapy at the level of blocking mRNA might be considered. Such an antisense RNA therapy should include removal of patient's bone marrow, administration of the gene for constitutive expression of an antisense RNA for the bcr — c-abl fusion gene into the myeloid stem cells and reinjecting the engineered marrow into the patient. Such an approach, comparable to autologous BMT, will have the advantages of absence of graft rejection and possibility of 100% remission. The possible nature of the gene construct for such an antisense RNA therapy is discussed.

Introduction

Though CML was the first leukemia to be diagnosed and treated, it is still a clinicians' dilemma, as the survival has not increased to more than 20–30 months as compared to the untreated patients (1). Extensive work has been done to obtain some insight into the pathology of the disease, so as to find the cure.

Stem cell assays and cell kinetic studies using several methods have shown that the chronic phase of the disease is characterised by the laterally expanded but growth factor responsive granulocyte-monocyte committed stem cells (CFU-GMs) which have a higher than normal proliferative index (2, 3).

This stem cell population and its progeny are minimally deviated from their normal untransformed counterparts except for the presence of the Ph' chromosome (4). The latter is the result of a reciprocal translocation between chromosomes 9 and 22 and in the process of this translocation proto-oncogenes c-cis and c-abl exchange their positions. In the normal granulocytes the product of the c-abl gene is a 145 kd protein having a very low tyrosine kinase activity (5, 6). In Ph', a fusion gene of bcr and c-abl is formed which codes for a protein of 210 kd having a very high tyrosine kinase activity (7). Since tyrosine kinases play a very important role in the growth factor induced signal transduction cascade, and since the

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bcr — c-abl fusion gene is the only consistent change in CML, it is reasonable to implicate the bcr — c-abl fusion in pathogenesis of the disease.

Three features which might, directly or indirectly, be the resultant of the bcr — c-abl fusion are: (a) loss of adhesiveness of Ph+ CFU-GM cells to the stromal cells (8); (b) elaboration of an IL-3-like synergistic activity which recruits multipotent stem cells into becoming CFU-GMs (3); and (c) production of a TGF-beta-type activity (8). These factors could, in turn, have serious effects on the normal residual haemopoietic stem cells, by disrupting the stromal 'niche' essential for their maintenance, finally leading to progression of the disease into a blastic crisis.

Bone marrow transplant (BMT)

All the above-mentioned features necessitate the use of a therapy which would correct the basic genetic defect in the stem cells in bone marrow. At the moment, BMT is the only procedure which offers a possible cure in CML. The International Bone Marrow Transplantation Registry (IBMTR) data shows that to date 63% patients have lived disease-free until 3 years after transplantation. However, the immediate complications of BMT are very high. The main drawback of BMT is that it can be offered only to that fraction of patients who are young and have HLA-matched donors.

Probable mode of action of p210

As discussed earlier, in CML the translocation of c-abl downstream of the bcr gene on chromosome 22 is the most consistent feature of the disease. On the other hand, circumstantial evidence suggests that Ph+ myeloid progenitor cells are somewhat deficient in their ability to adhere to stromal cells in marrow, which in the normal situation provides them with a micro-environment that allows control of proliferation and differentiation (8). It is probable that the molecule/s required for such an adhesion phenomenon are synthesized via a cascade. The normal p145 may be an integral part of such a cascade and, in CML, the aberrant p210 — perhaps due to a change in its substrate specificity — may bring about a break in the cascade, resulting in non-expression of the 'adherence molecule'. This might prompt the CFU-GM cells to move out of their micro-environment and continue to proliferate, responding constantly to growth factors. This hypothesis is diagrammatically represented in Figure 1.

Why antisense RNA therapy (ART)?

It has been shown that blocking of expression of the normal product of c-abl (p145) results in prevention of normal myeloid differentiation (9). This implies, conversely, that it may be essential to block the production of p210 in CML myeloid stem cells, or neutralize the protein by using a monoclonal antibody. The latter approach may not work since the 210 kd protein is not expressed on the cell surface, but is cytosolic.

The only viable approach left then is to neutralize the mRNA of the bcr — c-abl fusion gene by using an antisense therapy. The role of antisense RNA has been reviewed by Green et al (10) and possible therapeutic applications to AIDS and chronic active hepatitis have been suggested by Kohn et al (11). The effective sequestration of the transcript of translocated c-myc gene in Burkitt's lymphoma has been observed by Magarath (personal communication).

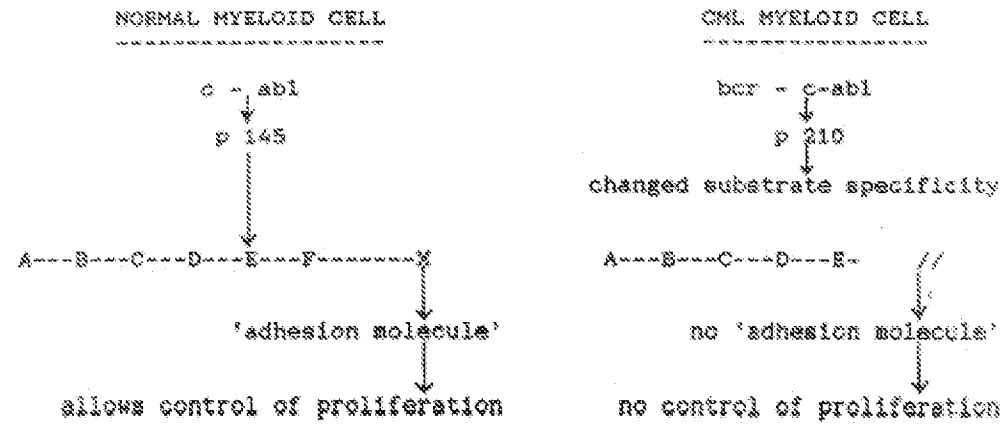

Fig. 1

Nature of gene construct

In the case of CML, the ART can be brought about by introducing into the CFU-GM cells a gene construct which constitutively transcribes a 16–20 nucleotide-long antisense message having a sequence complementary to the junctional region of bcr — c-abl mRNA (contributed by 8–10 nucleotides from 3' end of bcr fusion gene partner and the same length from 5' end of the c-abl fusion gene partner). Such an oligonucleotide antisense message will selectively block the bcr — c-abl mRNA but will not prevent the expression of the normal bcr and c-abl genes. This is because neither of the latter genes will have a sequence homology of more than 8–10 nucleotides with the antisense message, whereas the minimum homology requirement for a stable duplex function is 16–20 nucleotides. (Since the junctional region of the fusion gene varies depending upon whether exon 2 or 3 of bcr is the splice donor and whether exon 1a or 1b of c-abl is the splice acceptor, a selection of the correct antisense 'gene' will depend on the nature of junctional region of the patient to be treated). Such an antisense 'gene' can prevent the expression of p210, while permitting normal expression of p145. But merely neutralizing the aberrant mRNA may not be sufficient since only one functional normal allele might be incapable of synthesis of quantitatively normal amount of p145. To rectify this situation, additional copies of normal bcr and c-abl genes may be necessary.

Combining both the above approaches, it should be possible to construct a gene system having three components: (a) normal genes for bcr and c-abl; (b) antisense gene for the bcr — c-abl fusion gene; and (c) a drug resistance gene (see below). All these three components should be placed downstream of (i) a strong promoter sequence and (ii) a lineage–specific enhancer sequence (see below).

Such a construct, once introduced into the right type of target cell, should permit expression of the p145 while continuously preventing the expression of bcr — c-abl mRNA by constitutively transcribing the antisense message. Since Ph^- CML cells also show a fused bcr — c-abl sequence (12, and references therein), the above approach can be used in Ph^- CML patients too.

In order to increase the percentage of genetically engineered stem cells containing the functional construct, either the residual un-engineered cells can be eliminated by cytoablative therapy or the stem cells, manipulated ex vivo, can themselves be given a selective growth advantage (13, 14, 15). The former method poses a risk of long-term toxicity, but the latter one, where the drug resistance genes such as DHFR, MDR or Neo^b can be co-transferred with the exogenous gene of interest, has already proven to be effective in murine models.

Providing for all the above requirements, the possible gene construct would be as given in Figure 2.

Choice of method of gene transfer

Possible gene transfer methods and their limitations are reviewed in detail by Kohn et al (11). In brief, microinjection cannot be a method of choice because it is prohibitively time-consuming. Calcium phosphate-mediated gene transfer will also have to be ruled out because of low efficiency (10^{-6}–10^{-7}) and evidence of incorporation of multiple copies of the exogenous gene, which can be disastrous if it resulted into over-expression. This leaves the transfer via a retroviral vector as the only viable choice.

In this method, a replication-defective murine retrovirus, devoid of LTRs, can be used to carry the above-mentioned gene construct and the bone marrow CFU-GM cells can be infected ex vivo by this virus. Since this virus will be incapable of a second infection, the transferred gene construct will remain only in the target cells and their progeny. This will avoid horizontal spread of the gene construct. Secondly, there are no known human retroviruses which can complement murine retroviruses. This would preclude the possibility of rescuing of the infectious viruses. Finally, the insertional inactivation of the vital genes by the exogenous gene construct would be almost impossible as this would probably require simultaneous integration into both the copies of the cellular gene. This is supported by the fact that no viral infections lead to widespread cell dysfunction or death. Whatever dysfunction or death occurs in a few cells should not cause concern while correcting the defect in millions of cells. As an additional precautionary measure, an insertion of strong termination sequences at both

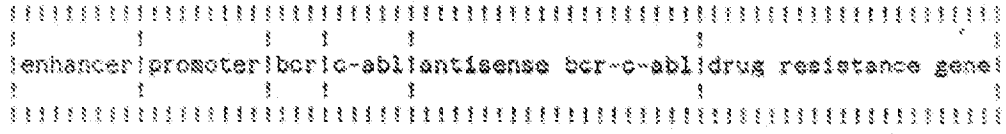

Fig. 2

ends of the vector should minimise the possibility of insertional inactivation.

The retroviral method of gene transfer has an additional advantage in that it has a very high efficiency and can transfer as much as 13 Kb long DNA into each one of a large number of cells.

Advantages of antisense RNA therapy

ART aims at genetically engineering the patients' own marrow cells ex vivo and re-introducing them into the body. Thus, it is comparable to autologous BMT, yet may ensure 100% remission, since the genetically engineered 'healthy' gene construct would never allow the residual defective stem cells — which are at a selective growth disadvantage to repopulate the marrow. Also, the few Ph⁻ CML cases can be treated by ART by virtue of the presence of the fused bcr — c-abl gene in their myeloid progenitors. Thus, absence of graft rejection and possibility of 100% remission in both Ph-bearing and Ph⁻ CML patients would be distinct advantages of the ART.

Loopholes and possible solutions

Although transfer and expression of exogenous genes in human bone marrow cells have been attempted (16, 17), only low and transient expression of these genes is seen. The transient expression indicates that, at least in these experiments, the recipient stem cells were committed to a later fraction of the lineage, and that a multipotent stem cell much earlier in the differentiation lineage would be the essential target. But again, especially in CML, the transfer of the gene construct into a very early pluripotent stem cell would lead to excessive production of the so-called 'corrected' myeloid cells while depleting drastically the number of the other haemopoietic cells. Thus, tissue-specific expression is not enough, but lineage-specific expression is essential. Such a sequence has not yet been identified, so the first task before the gene construction will be to identify this sequence.

In conclusion, although it may be a little early to undertake actual gene therapy for CML, the time is perhaps just ripe for consideration of possible gene constructs and of gene therapy.

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Inhibition of Proliferation by c-*myb* Antisense RNA and Oligodeoxynucleotides in Transformed Neuroectodermal Cell Lines[1]

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ABSTRACT

Transfection of a neuroblastoma cell line with expression vectors containing two different segments of human c-*myb* complementary DNA in antisense orientation yielded far fewer transfectant clones than did the transfection with the identical segments in sense orientation. In cell clones expressing c-*myb* antisense RNA, levels of the c-*myb* protein were down-regulated and the proliferation rate was slower than that of cells transfected with sense constructs or the untransfected parental cell line. Treatment of neuroblastoma and neuroepithelioma cell lines with a c-*myb* antisense oligodeoxynucleotide strongly inhibited cell growth. These data indicate a definite involvement of c-*myb* in the proliferation of neuroectodermal tumor cells extending the role of this protooncogene beyond the hematopoietic system. The availability of cell clones that transcribe c-*myb* antisense RNA provides a useful tool to study the involvement of other genes in the proliferation and differentiation of neuroblastoma cells.

INTRODUCTION

The c-*myb* protooncogene is the cellular homologue of the viral v-*myb* carried by avian myeloblastosis virus and E26 (1, 2), both of which transform hematopoietic cells with a distinct myeloid phenotype (1–3). The protein encoded by v-*myb* is localized in the nucleus (4, 5) and binds to DNA *in vitro* (6). The DNA-binding domain of the v-myb protein is composed of two imperfectly conserved 52-amino acid direct repeats located near the amino terminus (7) and corresponds to a truncated version of that found in chicken and mammalian c-myb proteins (8–10). The v-myb protein synthesized in bacteria binds specifically to the nucleotide sequence pyAACG/TG (11), and concatemers of this consensus sequence can confer v-*myb*-dependent inducibility to otherwise unresponsive promoters, suggesting that the v-myb protein acts as sequence-specific DNA binding transcription factor (12). Also, v-*myb* directly regulates the expression of a cellular gene, *MIM-1*, in chicken myeloblasts infected with an avian myeloblastosis virus temperature-sensitive mutant (13). Nuclear localization and DNA binding activity are necessary but not sufficient for the oncogenic potential of *myb* (14). The c-*myb* protooncogene and v-*myb* share several biochemical and functional properties, including nuclear localization, DNA binding, and transcriptional regulator activity (15). These properties appear to be important in transformation of chicken myeloid cells (16).

It has long been suggested that c-*myb* expression is linked to proliferative and differentiative processes in the hematopoietic system (17, 18). Antisense c-*myb* oligodeoxynucleotides block normal hematopoiesis *in vitro* (19). More recently, the need for c-*myb* expression on fetal liver hematopoiesis has been demonstrated in transgenic animals in which the c-*myb* gene has been inactivated by homologous recombination (20).

Elevated c-*myb* expression has been demonstrated in human leukemia cells (21) the *in vitro* proliferation of which has been shown to be *myb*-dependent (22). Several solid tumors of different embryonic origin such as colon carcinoma (23), small cell lung carcinoma (24), teratocarcinoma (25), and neuroblastoma (26) also demonstrate c-*myb* expression.

NB[4] is a malignant childhood tumor thought to arise in migratory cells of the embryonal neural crest (26). NB is histopathologically indistinguishable from NE and the two malignancies are often considered as one entity. Nevertheless, Thiele *et al.* (26) have reported that the pattern of protooncogene expression differs in these tumors. In fact, N-*myc* expression is generally high in NB and its amplification correlates with tumor progression and aggressiveness (27), whereas NE generally does not express N-*myc* (27). On the contrary, c-*myc* expression is high in NE but low in NB. However, both NE and NB express c-*myb*. NB cell lines induced to differentiate by retinoic acid demonstrate a rapid and sharp decrease in c-*myb* expression due to a decreased transcription rate rather than instability of c-*myb* mRNA (28). The temporal relationship between the levels of c-*myb* mRNA and the differentiative and proliferative processes occurring in NB makes c-*myb* a possible candidate for a key role in the proliferation and/or differentiation of neuroectodermal tumors.

In the present study we evaluated the effects of c-*myb* down-regulation in NB and NE cell lines by transfection of expression vectors carrying different domains of the c-*myb* cDNA in antisense orientation and by exposing cell cultures to antisense oligodeoxynucleotides.

MATERIALS AND METHODS

Cloning of Antisense and Sense c-*myb* Vectors. *Sst*II-*Eco*RI (DNA-binding domain) and *Bam*HI-*Bam*HI (3′ untranslated region) fragments were obtained from clone pMbm I dihydrofolate reductase (18) containing a full-length human c-*myb* cDNA. After end-repair with Klenow enzyme (Promega, Madison, WI), the fragments were cloned in the polylinker region of pRc/CMV vector (Invitrogen, San Diego, CA) as described (29). Sense and antisense orientation of the cloned fragments was determined by restriction analysis.

Cell Lines and Transfection. Neuroblastoma cell line LAN-5 (30) was grown in RPMI 1640 (Sigma, St. Louis, MO) supplemented with fetal bovine serum (Sigma). Cell lines SK-N-SH and SK-N-MC (31) were grown in minimal essential medium (GIBCO, Grand Island, NY) with 10% fetal bovine serum.

DNA transfections in LAN-5 cells were performed by the calcium phosphate precipitation technique according to standard procedures (29). Briefly, cells were seeded at a density of 10^6/dish and 48 h later were exposed to plasmid DNA (pRc/CMV) at 20 μg/plate for 6 h. After

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[4] The abbreviations used are: NB, neuroblastoma; NE, neuroepithelioma; cDNA, complementary DNA; PBS, phosphate-buffered saline; CMV, cytomegalovirus; RT, reverse transcriptase; PCR, polymerase chain reaction.

2 days cells were placed in medium containing the antibiotic G418 (Sigma; 400 μg/ml) and transfectant clones were isolated 3 weeks later.

Oligodeoxynucleotides. Unmodified deoxynucleotides were synthesized on an Applied Biosystem 380B DNA synthesizer by means of β-cyanoethylphosphoramidite chemistry. Oligodeoxynucleotides were purified by ethanol precipitation and multiple washes in 70% ethanol. Nucleotide (nt) numbers and codon positions for each c-*myb* oligodeoxynucleotide refer to the published human c-*myb* cDNA (10).

The primers for the PCR to control integration of the antisense inserts in the 5′ and 3′ antisense clones and to detect antisense RNA transcription are

CMV1: 5′ AATGGGAGTTTGTTTTGGCACCAA3′ nt 699–722 of hRc/CMV-*myb*-1: 5′ TGCCAAGCACTTAAAGGGGAGAAT3′ nt 467–490
myb-2: 5′ AACTTGTTTGGGAGACTCTGCATT3′ nt 2959–2983
myb-3: 5′ GCTGGCACTGCACATCTGTT3′ complementary to nt 333–352
myb-4: 5′ GCTGGCACTGCACATCTGTT3′ nt 128–147
myb-5: 5′ CTGAAGAAGCTGGTGGAACAGAATG3′ nt 264–289
myb-6: 5′ CTAGCAGCATGTCTACAGGC3′ complementary to nt 2708–2729
myb-7: 5′ CCATGTGACATTTAATCCAG3′ nt 2496–2515
myb-8: 5′ GCTCATTTATGGTTAATGAC3′ nt 2525–2544

The c-*myb* sense oligodeoxynucleotide used in cell cultures was 5′GCCCGAAGACCCCGGCAC3′ corresponding to codons 2–7. The c-*myb* antisense was 5′GTGCCGGGGTCTTCGGGC3′, complementary to the codons 2–7.

RNA Extraction and RT-PCR Analyses. Total RNA was prepared by acid guanidinium thiocyanate-phenol-chloroform extraction (32). RNA for RT-PCR analysis to detect c-*myb* antisense RNA in transfected clones was treated with RNase-free DNase I (Promega). Reverse transcription, PCR analysis, and hybridization to the specific probes were carried out as described (33). Plasmid integration was determined by PCR after isolating genomic DNA as described (29).

Immunocytochemical Analysis. LAN-5 cells were seeded in Labtek chamber slides (NUNC, Naperville, IL) at a density of 5×10^3 cells/cm^2. After 48 h, cells were rinsed in PBS, fixed in 4% paraformaldehyde in PBS for 10 min at 4°C, permeabilized in PBS containing 0.01% Triton X-100 for 5 min at 4°C, and treated with 1% bovine serum albumin (Sigma) for 30 min at room temperature. Incubation with sheep polyclonal antibody to c-myb protein (Cambridge Research Biochemicals, Valley Stream, NY) (34) at 1:100 dilution and immune sheep serum as control was carried out for 18 h at 4°C. After extensive washings in PBS, slides were treated with peroxidase-labeled rabbit anti-sheep IgG antibody (KPL, Gaithersburg, MD) and stained with an immunocytochemical staining kit (KPL).

RESULTS

Cloning Efficiency and Proliferation of a Human Neuroblastoma Cell Line Expressing c-*myb* Antisense Transcripts. Two different regions of the human c-*myb* cDNA were cloned in antisense orientation in the expression vector pRc/CMV in which the CMV promoter-enhancer (35) drives the transcription of the cloned genes and the SV40 promoter drives transcription of the gene encoding the G-418 resistance used to select transfected cells. Transient transfection assays to compare the efficiency of the Rous sarcoma virus, SV40, and CMV promoters in LAN-5 neuroblastoma cells showed that the CMV had highest activity in driving the transcription of a reporter gene.[5]

Neuroblastoma cell line LAN-5 which expresses c-*myb* mRNA constitutively (26, 28) was transfected with plasmids expressing antisense and sense c-*myb* transcripts. Both size and number of G418 resistant clones were reduced in the transfections with constructs expressing the 5 or 3′ antisense *myb* sequences as compared to those expressing the sense transcripts (Fig. 1). On average, the cloning efficiency of 5′ antisense-*myb* transfectants was reduced 66% (from 62 to 70%) as compared to 5′ sense-*myb*; for the 3′ antisense-*myb* transfections, the average reduction was 91% (from 84 to 97%) as compared to 3′ sense-*myb* (mean of 3 separate experiments).

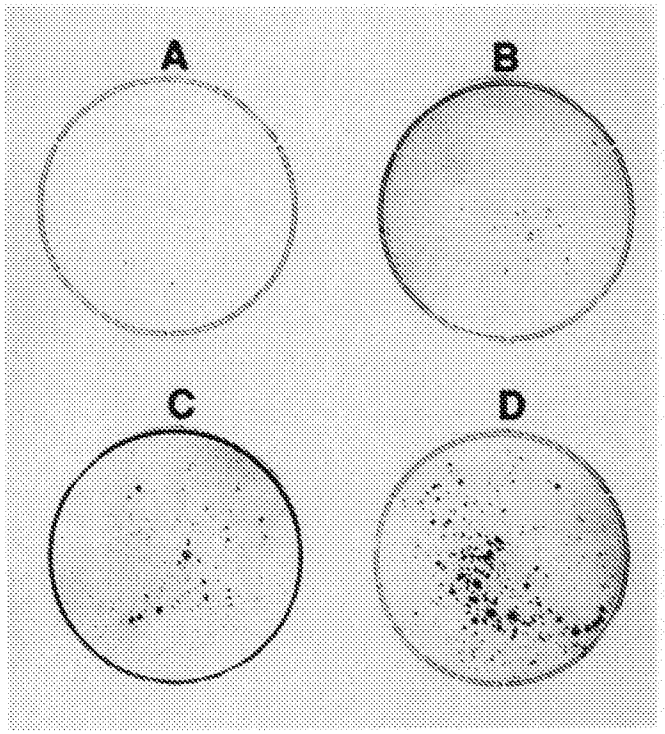

Fig. 1. Cloning efficiency of LAN-5 cells transfected with plasmids transcribing c-*myb* cDNA in the sense and antisense orientation. *A*, 5′ antisense *myb*; *B*, 3′ antisense *myb*; *C*, 5′ sense *myb*; and *D*, 3′ sense *myb*. Results are representative of 3 separate experiments.

Twelve single clones were isolated from the 5′ and 3′ antisense *myb* transfectants and the integration of each construct was determined by PCR. Primers for PCR were chosen in order to amplify 267 base pairs upstream of the *Not*I cloning site in addition to 208 base pairs of 5′ antisense-*myb* and 219 bp of 3′ antisense *myb*. Fig. 2 shows a diagram illustrating PCR strategies with primers and probes used for the analysis of the clones. All 3′ antisense *myb* clones showed the expected amplified fragment (486 base pairs) (Fig. 3*A*), whereas several 5′ antisense *myb* clones (Fig. 3*B*, *Lanes 2*, *4*, *7*, and *8*) did not,

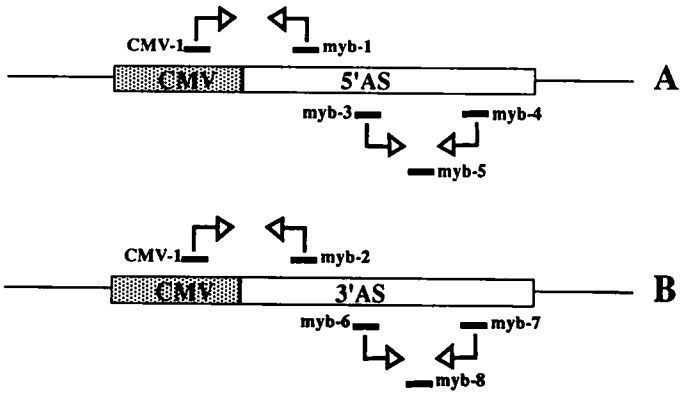

Fig. 2. PCR strategies to detect vector integration and transcription of antisense RNA in antisense transfectants. In *A*, CMV-1 and *myb*-1 are the PCR primers to determine the integration of the 5′ antisense (*AS*) clones; *myb*-3 and *myb*-4 are the primers for RT-PCR; and *myb*-5 is the probe to detect antisense RNA transcripts in 5′ antisense clones. In *B*, CMV-1 and *myb*-2 are the PCR primers to control the integration of the 3′ antisense clones; *myb*-6 and *myb*-7 are the RT-PCR primers; and *myb*-8 is the probe for antisense RNA detection in 3′ antisense clones.

[5] G. Raschellà et al., unpublished observations.

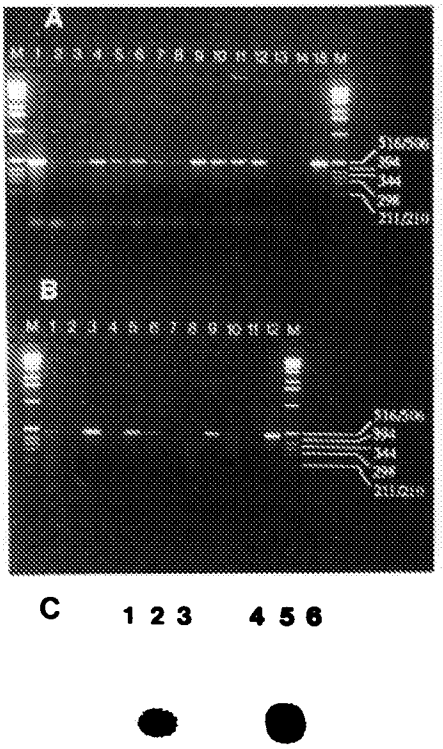

Fig. 3. Detection of construct integration and antisense transcripts by PCR analysis. *A*, 3′ antisense *myb* clones. *M*, size marker. *Lane 1*, pooled transfectant clones; *Lanes 2–12*, individual clones; *Lane 13*, transfectant clone containing the pRc/CMV vector (negative control); *Lane 14*, no DNA; *Lane 15*, DNA from vector 3′ antisense *myb* (positive control). *B*, 5′ antisense *myb* clones. *M*, size marker. *Lane 1*, pooled transfectant clones; *Lanes 2–9*, individual clones; *Lane 10*, transfectant clone containing the pRc/CMV vector (negative control); *Lane 11*, no DNA; *Lane 12*, DNA from vector 5′ antisense *myb* (positive control). *C*, hybridization analysis of antisense RNA transcripts in transfectant clones. *Lane 1*, RT-PCR without RNA; *Lane 2*, RT-PCR of 5′ antisense clone in *B*, *lane 9*; *Lane 3*, PCR from RNA of the same clone; *Lane 4*, RT-PCR without RNA; *Lane 5*, RT-PCR of 3′ antisense *myb* clone, in *A*, *Lane 7*; *Lane 6*, PCR from RNA of the same clone. Primers and probes are as described "Materials and Methods."

possibly because of rearrangement or deletion involving the antisense insert preventing the transcription of the antisense RNA. Accordingly, the inhibition of LAN-5 cell proliferation resulting from transfection with the 5′ antisense *myb* construct might be underestimated. Clones positive for integration of the 5′ antisense construct showed the expected amplified 475-base pair fragment.

Transcription of antisense c-*myb* RNA was confirmed in RT-PCR experiments (Fig. 3*C*). Total RNA from transfectant clones was extracted and treated with RNase-free DNase I before the RT-PCR reaction. Primers were designed to amplify a 224-base pair product for 5′ antisense *myb* clones and a 231-base pair product for 3′ antisense *myb* clones (Fig. 2). After size fractionation on an agarose gel and transfer to a nylon membrane, the amplified products were hybridized to specific probes for unambiguous identification; antisense c-*myb* RNA transcripts were clearly detected in both 5′ and 3′ antisense-*myb* transfectants (Fig. 3*C*). Most of the antisense transfectants do not show evident morphological alterations. All tested antisense clones retain the capability to differentiate toward a neural phenotype under the effect of retinoic acid (not shown). A more detailed phenotypic characterization of the transfectant clones is now in progress.

Comparison of growth curves for 3′ and 5′ antisense-*myb* clones which were found positive for integration of the constructs (Fig. 4) revealed consistently slower proliferation rates and generally lower growth plateaus than those of the LAN-5 parental cells and sense controls.

Immunocytochemical analysis using an anti-*myb* polyclonal antibody indicated the clear presence of myb protein in the nucleus of the LAN-5 parental cells and the 5′ and 3′ sense controls (Fig. 5, *A–C*), whereas myb protein was barely detectable in the 5′ and 3′ antisense transfectants (Fig. 5, *D* and *E*).

Effect of c-*myb* Antisense Oligodeoxynucleotides on Growth of Neuroblastoma and Neuroepithelioma Cell Lines. To further examine the role of c-*myb* in the proliferation of neuroectodermal tumors, we inhibited c-*myb* expression using antisense oligodeoxynucleotides and analyzed the effects of this

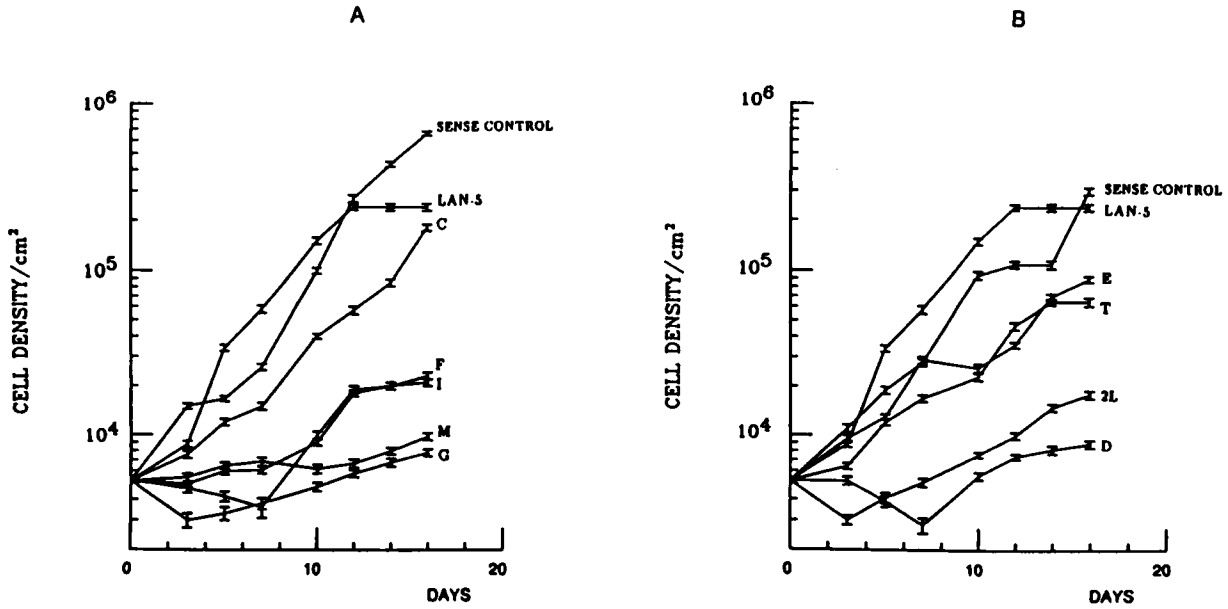

Fig. 4. Growth curves of antisense-*myb* transfectants. *A*, 3′ antisense *myb*; *B*, 5′ antisense *myb*. Values are the mean of two independent experiments. The sense-*myb* control curve in each panel is derived from the mean values using three different 5′ (*A*) and 3′ (*B*) sense clones. Each sense clone had a comparable growth rate. Initial seeding density was 5×10^3 cells/cm². *Letters*, individual clones. *Bars*, SE.

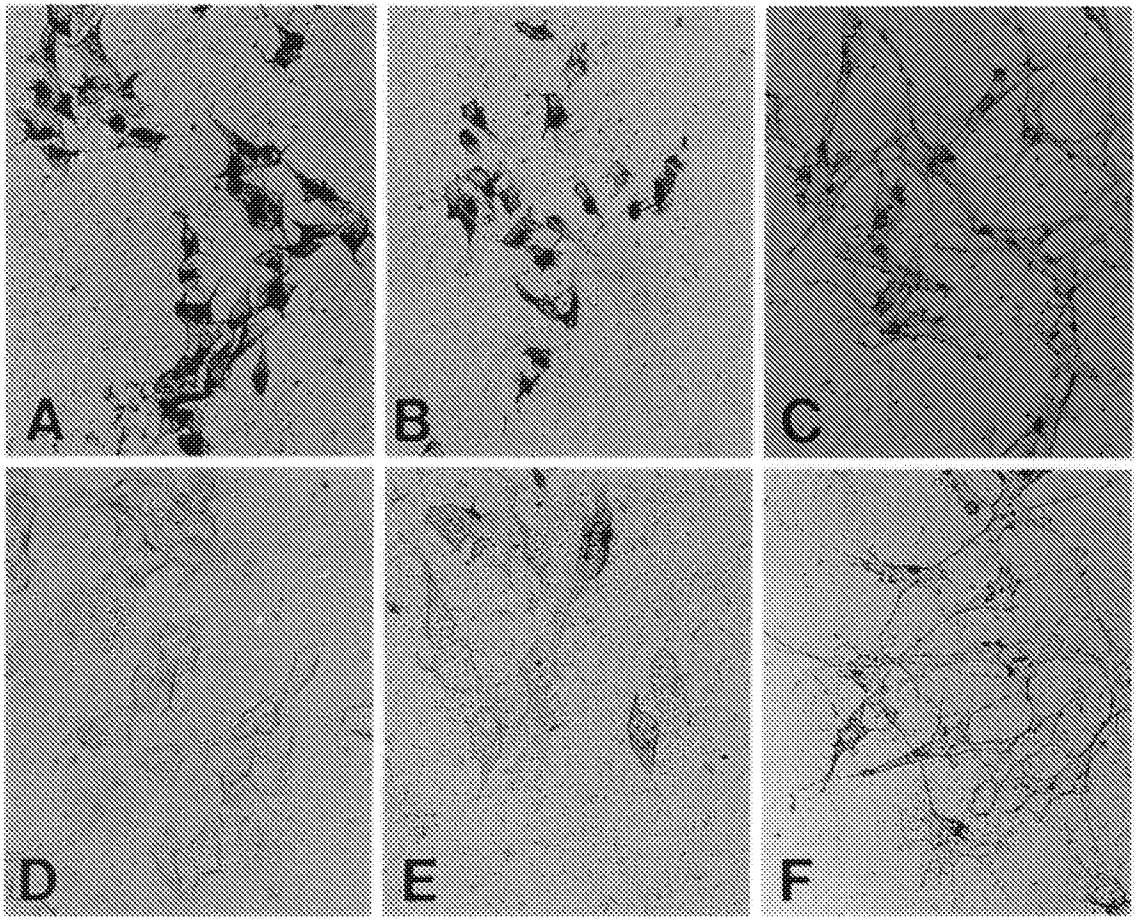

Fig. 5. Expression of c-*myb* protein in antisense clones. Cells were treated with an anti-*myb* specific polyclonal antibody (*A–E*) or nonspecific preimmune serum (*F*) as described in "Materials and Methods." *A*, LAN-5; *B*, 5′ sense *myb*; *C*, 3′ sense*myb*; *D*, 5′ antisense *myb*; *E*, 3′ antisense *myb*; and *F*, 3′ sense *myb* treated with immune serum. × 200.

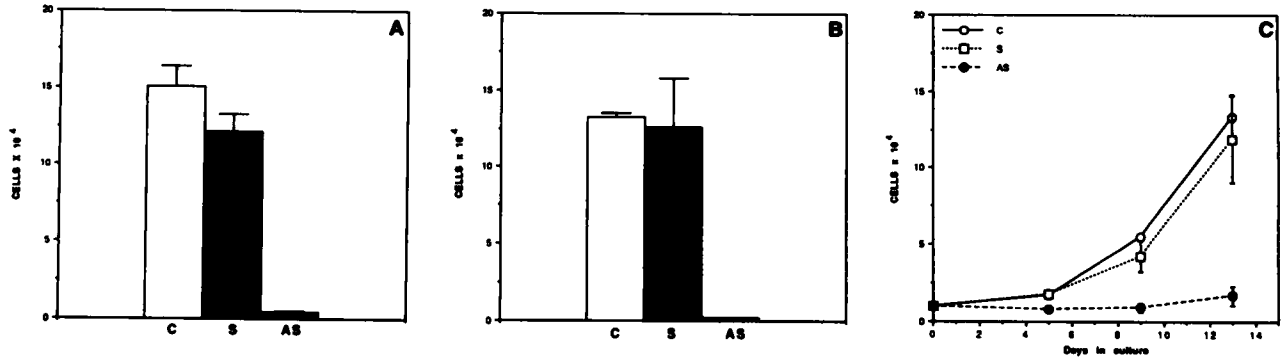

Fig. 6. Effect of c-*myb* antisense oligodeoxynucleotide on cell growth of neuroblastoma and neuroepithelioma cell lines. SK-N-SH (*A*), SK-N-MC (*B*), and LAN-5 (*C*) cells were untreated (*C*), sense-treated (*S*) and antisense-treated (*AS*). Cells were counted after 7 days for SK-N-SH (*A*) and after 9 days for SK-N-MC (*B*). LAN-5 (*C*) cells were counted at days 0, 5, 9, and 13 of culture. Cell count values, mean ± SD (*bars*) of experiment performed in triplicate.

inhibition on the growth of neuroblastoma cell lines LAN-5 and SK-N-SH and neuroepithelioma cell line SK-N-MC (31). In a typical experiment, 1×10^4 cells were seeded in the presence of antisense or sense oligodeoxynucleotides (80 μg/ml at 0 h, 40 μg/ml after 18 h, and 40 μg/ml after 36 h). Cells were counted after 7 or 9 days. As shown in Fig. 6, antisense c-*myb* oligodeoxynucleotide treatment resulted in almost complete growth inhibition in all three cell lines. To determine whether this inhibition correlated with c-*myb* transcript levels, total RNA was extracted from each tumor cell line 24 h after exposure to 120 μg of c-*myb* oligodeoxynucleotides and c-*myb* expression was measured by RT-PCR; c-*myb* mRNA was barely detectable in antisense-treated cells, but abundantly expressed in sense-treated and untreated cells (Fig. 7).

DISCUSSION

We have shown that down-regulation of c-*myb* expression exerts a strong inhibitory effect on the proliferation of neuroectodermal tumor cells. Two different strategies were used in our work. Neuroblastoma cell line LAN-5 was transfected with vectors carrying two different segments of the human c-*myb* cDNA in the antisense orientation and transfection efficiency was assayed; the yield of transfectants was dramatically reduced

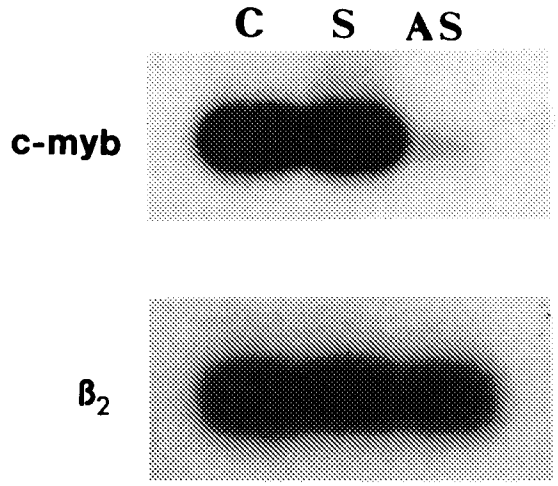

Fig. 7. Expression of c-*myb* mRNA in LAN-5 cells exposed to c-*myb* oligodeoxynucleotides. LAN-5 cells (10^5/ml) were untreated (*C*) or exposed to ~80 μg/ml of c-*myb* sense (*S*) or antisense (*AS*) oligodeoxynucleotides at time 0. After 12 h a second dose (40 μg/ml) was added. Cells were harvested 12 h later. Total RNA was isolated and divided into two aliquots that were separately amplified by RT-PCR with c-*myb*- and β_2-microglobulin-specific primers as described (44). The resulting cDNAs were hybridized to specific ^{32}P-end-labeled probes as described (29). Results are from a representative experiment.

when either antisense vector was used as compared to their sense controls. Although the isolated antisense transfectants are heterogeneous in their growth rate, they show consistently a slower proliferation as compared to sense controls. Furthermore, they transcribe antisense c-*myb* mRNA and have a marked reduction of myb protein synthesis as indicated by immunocytochemistry. Together these data indicate that the slower proliferation of the isolated antisense clones is due to down-regulation of c-*myb* expression caused by antisense RNA production.

The second strategy involved exposure of neuroblastoma and neuroepithelioma cell cultures to a c-*myb* antisense oligodeoxynucleotide in order to suppress c-*myb* mRNA expression. Again, the same inhibitory effect on the proliferation rate of these cells was observed. The rescue of antisense stable transfectants is in apparent contrast with the nearly complete growth inhibition resulting from the exposure of neuroectodermal tumor lines to c-*myb* antisense oligodeoxynucleotides. Most likely these findings rest in the low level of antisense c-*myb* RNA transcribed by the transfectant clones and detectable by the sensitive RT-PCR technique but insufficient to completely block the function of c-*myb* mRNA. In this regard, Cotten *et al.* (36) have demonstrated the requirement for an antisense RNA-:target mRNA ratio of 6:1 to completely abolish the function of protein U7. Nevertheless, the slow proliferation rate of the c-*myb* antisense transfectants indicates that even incomplete down-modulation of c-*myb* has a readily detectable effect on cell growth.

In neuroblastoma several structural abnormalities such as the deletion of the short arm of chromosome 1 (del1p32-pter), double-minute chromosomes and homogeneously staining regions are frequent findings (37–39) and have been associated with the development and the progression of this neoplasia (40). Furthermore, N-*myc* gene amplification has been correlated with advanced clinical stages and poor clinical outcome (27, 41). However, the proliferative activity of neuroblastoma has not been clearly associated with a distinct pattern of altered gene expression.

Two recent reports describe the block of N-*myc* expression by means of antisense RNA and oligodeoxynucleotides (42, 43). In both cases neuroblastoma proliferation was only partially affected by N-*myc* down-regulation and was probably secondary to the induction of differentiative processes. In addition, a large percentage of terminal neuroblastomas do not show amplification or detectable expression of N-*myc*. Together, those data suggested that other gene activities besides N-*myc* are involved in the proliferation of neuroblastoma cells. The inhibitory effect on cell growth obtained by abolishing the expression of c-*myb* strongly suggests the involvement of this protooncogene in the regulation of neuroblastoma cell proliferation. Moreover the findings reported here, provide direct evidence for the essential role of c-*myb* in nonhematopoietic tissues, perhaps through its effects on the expression of genes directly involved in DNA synthesis and cell cycle progression. The stable transfectant cell lines that express antisense c-*myb* RNA should prove useful in evaluating the possible cooperation of c-*myb* and other genes in regulating proliferative and differentiative processes in neuroblastoma, which, in turn, may lead to the development of an antisense-based therapy of these neoplastic disorders.

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